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Quantitative characterization of *O*-GalNAc glycosylation

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O-GalNAc type glycosylation is an abundant and complex protein modification. Recent developments in mass spectrometry resulted in significant success in quantitative analysis of *O*-GalNAc glycosylation. The analysis of released *O*-GalNAc type glycans expanded our horizons of understanding the glycome of various biological models. The site-specific analysis of glycosylation micro-heterogeneity of purified proteins opened perspectives for the improved design of glycoprotein therapeutics. Advanced gene editing and chemical technologies applied to *O*-glycoproteomics enabled to identify *O*-GalNAc glycosylation at unprecedented depth. Progress in the analysis of intact glycoproteins under native and reduced conditions enabled the monitoring of glycosylation proteoform variants. Despite of the astonishing results in quantitative *O*-GalNAc glycoproteomics, site-specific mapping of the full *O*-GalNAc structural repertoire in complex samples is yet a long way off. Here, we summarize the most common quantitative strategies in *O*-GalNAc glycoproteomics, review recent progress and discuss benefits and limitations of the various approaches in the field.

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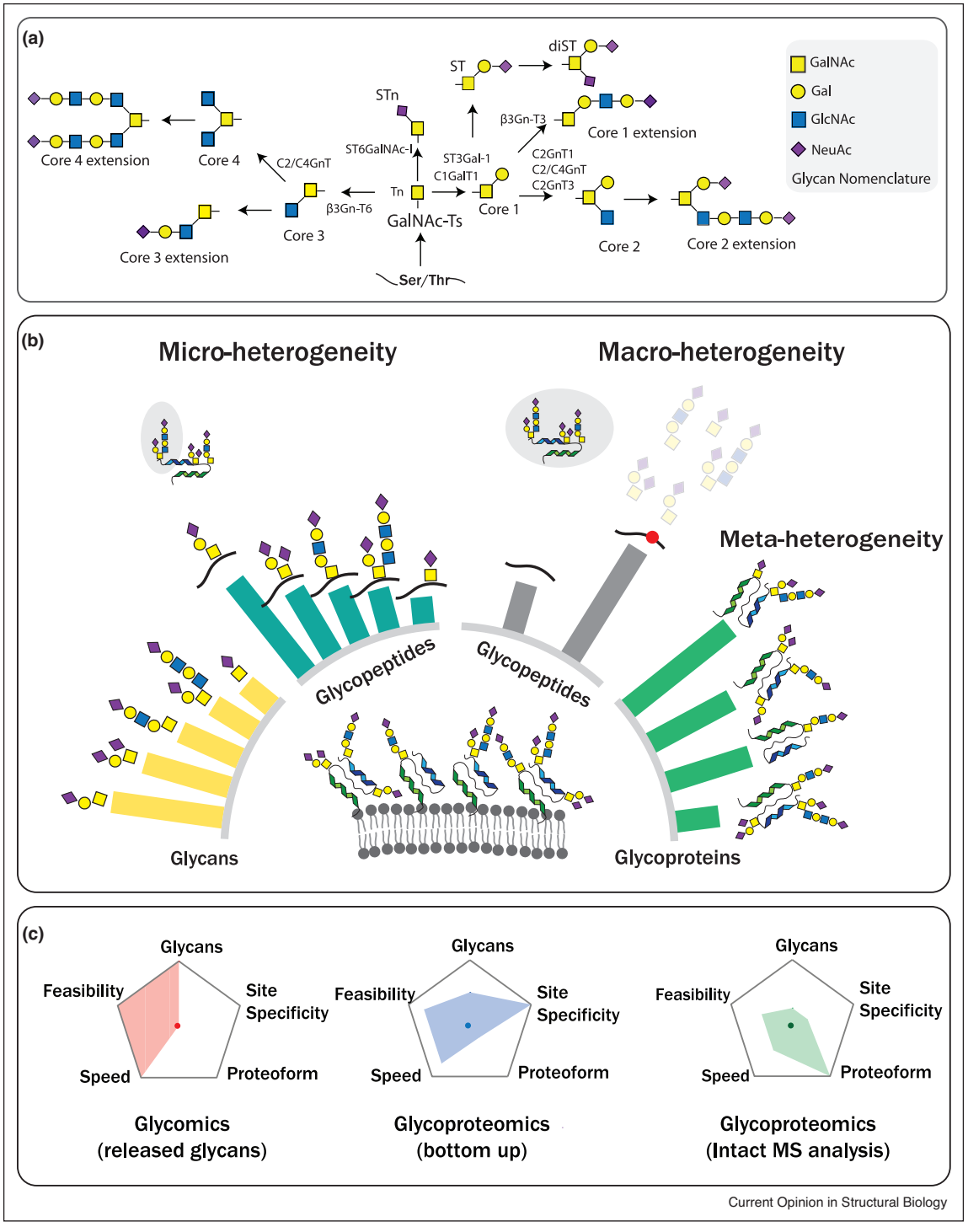
Introduction

In all living cells, the majority of proteins and lipids passing through the secretory pathway undergo the post-translational modification called glycosylation [1••]. Glycosylation plays a functional role in a range of biological processes, such as receptor activation, cell–cell interaction, cell adhesion, endocytosis, molecular trafficking, and protein clearance [2]. Alterations in cellular glycosylation are associated with disease states, including various types of cancer [3–6]. The glycosylation pathway involves the attachment of a monosaccharide or oligosaccharide moiety to a protein or lipid. This process is orchestrated by a wide variety of specific enzymes. Recently, 169 glycosyltransferases genes regulating the human glycome were summarized based on the glycosylation type they regulate [7•]. Sixty enzymes are related to the initiation step of distinct glycan types, including protein *N*-linked and *O*-linked glycosylation, lipid glycosylation and glycosylaminoglycans. Additionally, immediate core extension and branching are governed by 57 enzymes and another 52 enzymes are reported to perform pathway-nonspecific elongation, branching and end capping. The large family of glycosylation enzymes contribute to the complexity and heterogeneity of the glycome repertoire and drive the great diversity and multi-functionality of the proteome [8]. In short, we now possess intimate knowledge of genes that serve either distinct or multiple glycosylation pathways. These genetic insights coupled with advanced analytical approaches to measure glycoconjugates offer an unprecedented insight into the functional aspects of glycosylation.

Mucin-type *O*-glycosylation (also known as *O*-GalNAc glycosylation) is essential in many biological processes and one of the most diverse and differentially regulated forms of protein modifications. Whereas most glycan types are initiated by a limited number of enzymes, the initiation of *O*-GalNAc type glycosylation is regulated by 20 isoenzymes (GalNAc transferases; GalNAcTs) with varying protein backbone or domain specificities. The 20 GalNAcTs control the attachment of the core GalNAc residue to the hydroxyl group of a serine or threonine (and to a lesser extent, tyrosine) side chain [1••].The expression levels of those 20 isoenzymes in a cell determine the localization and site occupancy of *O*-GalNAc glycosylation on the proteins going through the secretory pathway. The large number of GalNAcTs suggest that these enzymes have the ability to modulate the *O*-glycoproteome in a differential way and are likely to dictate the function of the glycosylated protein [9,10]. Furthermore, the elongation and branching of the *O*-GalNAc cores by another set of glycosyltransferases adds an additional layer of functional complexity to *O*-GalNAc glycosylation [11] (Figure 1a). The overall combinatorics of glycosylation events on multiple sites, together with the *O*-glycan structural diversity, produce a high number of glycoproteoforms of different abundances. These are important to characterize when studying biological systems [12].

Figure 1



Overview of quantitative strategies in *O*-GalNAc glycoproteomics.
(a) The most common steps of *O*-GalNAc glycosylation pathway with the enzymes involved in extension and branching of *O*-GalNAc structures. **(b)** Schematic presentation of quantitative analysis at three different levels (glycans, glycopeptides and glycoproteins). **(c)** Comparison of analytical potential of glycomics, bottom up glycoproteomics and intact MS glycoproteomics approaches.

Strategies for quantitative *O*-GalNAc glycosylation analysis

Because of the high diversity and complexity of glycan moieties the characterization of glycoproteoforms is not trivial. Glycoproteins can be present in multivariant forms with different heterogeneity levels and these can be addressed *via* various analytical approaches, depending on the exact biological question asked. For example, the structural analysis of all glycans in a biological sample (e.g. a biofluid, tissue or cell type) can be performed after the liberation of the *O*-GalNAc glycans from their protein carriers. This allows to study global glycosylation perturbations in biological systems. However, in such analyses the connection with corresponding proteins is typically lost and the profiles obtained are highly affected by specific glycoprotein abundances (Figure 1b). A more protein-specific approach, where glycans and proteins are analyzed simultaneously, is reached in glycopeptide-centric workflows. Such glycoproteomic approaches aim to address the micro-heterogeneity and macro-heterogeneity of the glycosylation landscape, representing the site variability and occupancy, respectively (Figure 1b). While glycoproteomics via glycopeptides already results in highly information-rich data, no connection can be made between the occupancy levels and micro-heterogeneity of the different glycosylation sites on the same protein molecule. This information level was recently termed as meta-heterogeneity [13] and can be assessed studying intact glycoproteins. Such intact mass analysis require highly advanced technologies and have currently a limited capability to perform in-depth structural analysis of glycans and/or to perform the analysis in a site-specific way. Depending on the specific study requirements, such as deep structural glycan identification, site-specific glycomics, proteoform characterization, speed of analysis or technical feasibility, all these approaches have both advantages and disadvantages (Figure 1c).

Quantitative mapping of released *O*-GalNAc glycans

The chemical or enzymatic release of glycans from glycoconjugates before their analysis is a broadly used and powerful approach for the in-depth characterization of glycan structures present in a biological sample [14•,15]. Prior to mass spectrometry (MS)-based or fluorescence-based profiling, often a derivatization step is introduced, to stabilize specific glycan features and/or enhance their ionization or fluorescence. These include the stabilization of sialic acids (*via* esterification, amidation or permethylation) [16], the enhancement of the hydrophobicity of the analytes (*via* permethylation or reducing end labeling) [17] and/or the introduction of a fluorophore (*via* reducing end labeling) [18]. Powerful approaches for the label-free relative quantification of *O*-GalNAc glycans are porous graphitized carbon (PGC)-liquid chromatography (LC)–MS of reduced glycans [19–21] and matrix-assisted laser desorption/ionization (MALDI)-MS or LC–MS of permethylated glycans [22–24]. These approaches provide a wealth of structural information and have shown their potential for the profiling of *O*-GalNAc glycans derived from isolated proteins, biofluids, cell lines and tissues [25–27]. With these strategies, total area normalization to the sum of all analytes is used to obtain relative glycan levels.

Absolute quantification of individual analytes or multiplexing of samples by MS can be obtained when isotope labels are introduced to the glycans. Prominent examples are isotope labeled amine tags such as 2-aminobenzoic acid [28] and INLIGHT [29] for duplex analysis or the aminoxy-based tandem mass tag for six-plex analysis [30]. While these strategies have shown great potential for the quantification of *N*-glycans, the developments lack behind for *O*-GalNAc glycans. The main reason for this is that, while *N*-glycans can be released with the broadly specific enzyme PNGase F, the release of *O*-GalNAc glycans relies heavily on reductive β-elimination, preventing the functionalization of the reducing end [14•]. However, recent developments start to enable the minimally destructive, non-reductive chemical release of glycans. For example, by the direct addition of a reducing end label during the release [31] or the use of an organic superbase that prevents glycan peeling [32]. Alternatively, *O*-GalNAc amplification techniques, introducing benzene-conjugated GAlNAc precursors to cell cultures, allow to provide an insight into the *O*-GalNAc glycosylation capabilities of a cellular system [33•]. All these strategies have the potential to accelerate quantitative *O*-GalNAc glycan analysis in the near future.

Quantitative *O*-GalNAc glycoproteomics at glycopeptide level

Bottom up approaches for glycoproteomics require the digestion of proteins with proteolytic enzymes (e.g. trypsin or chymotrypsin) and subsequent analysis of the resulting (glyco)peptide mixture by reversed-phase LC–MS [34,35,36•,37••]. When moderate complex samples of isolated glycoproteins are assessed, *O*-GalNAc glycopeptide analysis can be performed in a high-throughput manner, as was shown for the site-specific *O*-glycan analysis of, for example, immunoglobulin A, C1 inhibitor and erythropoietin (EPO) [38–40]. In these examples the full complexity of the glycan microheterogeneity was assessed and an estimation of site occupancy could be made as no enrichment of glycopeptides was performed before analysis. However, such detailed, site specific, glycan characterization is still unachievable for complex samples, especially in a high throughput manner.

A large advancement towards proteome wide *O*-GalNAc localization derived from the introduction of so called SimpleCells wherein the knockout of exclusive C1GalT1 Core 1 synthase and the COSMC chaperone

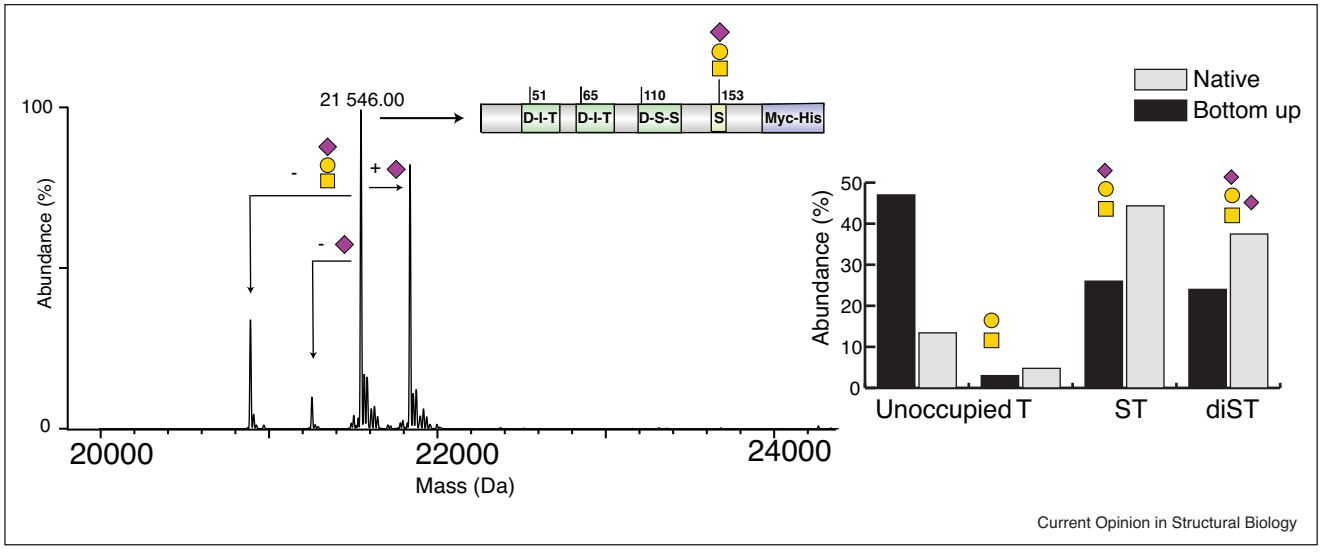
resulted in the expression of truncated Tn and in some cases STn *O*-glycan structures. Based on the enrichment of glycopeptides using lectin weak affinity chromatography (LWAC), this technology allowed the mapping of thousands of glycopeptides derived from complex biological mixtures [41]. One of the serious limitations of this approach is the necessity to enrich for glycopeptides to overcome the large difference in abundance between glycosylated (low) and non-glycosylated (high) peptides [42]. Since the enrichment removes non-modified peptides from the mixture, quantification of the corresponding site occupancy is hampered [43]. Despite these limitations, this strategy has shown its advances in the assessment of GalNAcT specificities. The SimpleCell glycoproteomics approach was combined with genetic manipulation of GalNAcTs to map the contributions of the individual enzymes to the glycoproteome [44•]. For instance, a recent report described how GalNAcT11 regulates kidney function through glycosylation of the endocytosis receptor megalin [45]. Another recent advancement towards differential *O*-GalNAc glycoproteomics came with the introduction of the 'bump-and-hole' engineered GalNAcTs that accept unnatural UDP-GalNAc analogues and uniquely tag their substrates [46•,47]. A large benefit of this approach is that the tagged substrates undergo full elongation of their Core 1 *O*-glycans, giving rise to ST and diST structures. However, this approach also requires *O*-glycopeptide enrichment, resulting in the loss of information of the actual *O*-glycosylation stoichiometry.

To overcome the high dynamic range between peptide and glycopeptide abundances in a biological samples and address *O*-GalNAc site occupancy, a promising solution was recently reported by Ye. *et al*. Here, a data independent (DIA) mass spectrometry approach for quantitative *O*-glycoproteomics was developed, enabling characterization of 269 *O*-glycopeptides carrying up to five different Core 1 *O*-glycans in a single shot experiment of unenriched human serum [48••]. Further improvements in DIA data analysis, including the inclusion of more elaborate *O*-glycan structures, library building, and spectral prediction, together with advancements in sample preparation such as (glyco)protein fractionation, represent a promising future avenue towards an unbiased large-scale quantitative *O*-glycoproteomics.

Quantitative *O*-GalNAc glycoproteomics at the intact protein level

Another emerging approach is quantification of *O*-GalNAc glycosylation at the intact protein level under native or reduced conditions. Native mass spectrometry was for instance used for the in-depth characterization of EPO glycosylation [49]. Since intact mass measurements provide information about total glycan composition of a studied protein and no site-specificity can be obtained with current technologies, the removal of *N*-glycans is required for confident *O*-GalNAc glycosylation profiling. In Figure 2 an illustrative example of PNGaseF treated EPO is provided, showing the unambiguous annotation and relative quantification of EPO *O*-glycosylation. Furthermore, unlike bottom up approaches where a lower

Figure 2



Intact MS characterization of Erythropoietin.
Deconvoluted intact MS spectrum of Erythropoieting is shown on the left where the most abundant peak corresponds to EPO with a ST antigen (illustrated in the inset graphic) and the neighboring peaks correspond to sequential addition or loss of denoted glycan residues. On the right a quantitative comparison of intact MS and glycopeptide based approach is shown.

ionization efficiency of glycopeptides as compared to their non-modified counterparts can lead to underestimation of *O*-glycosite occupancy, this issue is largely avoided under intact protein mass measurement as the hydrophilic properties of the *O*-glycans represent a minor fraction of the complete glycoconjugate (Figure 2). Additionally, a hallmark of mucin type *O*-glycosylation is that it usually occurs in dense clusters that are recalcitrant to peptide mapping methods and in this case native MS can provide insights about total number of occupied *O*-glycosites. For instance Wohlschlager *et al.* have characterized Etanercept, a highly glycosylated fusion protein formed from TNF-α receptor domain fused to the IgG_1 Fc domain [50•]. The resulting dimer contains 4 *N*-glycosylation sites and 26 *O*-GalNAc glycosylation sites. By measuring PNGase F and sialidase treated Etanercept the authors were able to demonstrate that the protein exhibits high heterogeneity in *O*-glycosite occupancy ranging from 14 to 23 occupied sites carrying Core 1 glycans. Using this information they were subsequently able to characterize and annotate sialylated Etanercept which has shown high heterogeneity in its sialylation profile carrying anywhere between 18–31 sialic acid residues. Finally, Yen *et al.* have combined native MS with clever enzymatic treatments to characterize the *O*-glycosylation profiles of the DC-SIGN carbohydrate recognition domain (CRD) [51•]. In their work they have demonstrated that by utilizing a combination of enzymatic treatments they were able to decipher the micro- and macroheterogeneity of DC-SIGN CRD as well as to differentiate between Core 1, Core 2 and Core 3 *O*-glycans. Furthermore, this approach enabled them to observe that the number of *O*-glycans attached to the glycoprotein, rather than the mass or elaboration of said glycans, correlates with the stability of DC-SIGN.

Conclusion

Over the past ten years, the analysis of *O*-GalNAc type protein glycosylation has seen many and large developments at all levels of analysis, assessing the micro-heterogeneity, macro-heterogeneity and even meta-heterogeneity of protein glycosylation. Still, the developments are lacking behind the developments made for *N*-glycosylation analysis in the same time span. For example, although a significant progress has been achieved for quantitative analysis of the released *O*-GalNAc glycans, the analytic methodologies are still awaiting for a robust, efficient and nondestructive method for *O*-GalNAc glycan release that allows functionalization of the reducing end of the glycan. Site-specific quantitative *O*-GalNAc glycoproteomics has already enabled many spectacular discoveries in the field addressing glycosylation microheterogeneity on various therapeutic glycoproteins and isolated plasma glycoproteins. However, these approaches are still limited for more complex samples by the high dynamic range between glycosylated and non-glycosylated peptides. Furthermore, unprecedented depth of *O*-glycosylation occupancy at the marcoheterogeneity level has been reached with the recent developments in intact MS analysis when studying isolated proteins. Despite of this success in quantitative analysis of *O*-glycoproteins, identification of full *O*-glycosylation repertoire at single glycosylation site, simultaneously with proteoform resolution is still an unattainable goal. To fully understand the functional role of *O*-glycosylation machinery we require further improvements in sample preparation and more advanced hardware apparatus for the analysis as well as more sophisticated software solutions for data processing.

Conflict of interest statement

Nothing declared.

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References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. •• Schjoldager KT, Narimatsu Y, Joshi HJ, Clausen H: **Global view of human protein glycosylation pathways and functions**. *Nat Rev Mol Cell Biol* 2020, **21**:729-749

A comprehensive detailed review about the glycosylation machinery, summarizing enzymes responsible for initiation, elongation, branching and capping *O*-glycan structures.

2. Varki A: **Biological roles of glycans**. *Glycobiology* 2017, **27**:3-49.

3. Gupta R, Leon F, Rauth S, Batra SK, Ponnusamy MP: **A systematic review on the implications of O-linked glycan branching and truncating enzymes on cancer progression and metastasis**. *Cells* 2020, **9**.

4. Mereiter S, Balmana M, Campos D, Gomes J, Reis CA: **Glycosylation in the era of cancer-targeted therapy: where are we heading?** *Cancer Cell* 2019, **36**:6-16.

5. Peixoto A, Relvas-Santos M, Azevedo R, Santos LL, Ferreira JA: **Protein glycosylation and tumor microenvironment alterations driving cancer hallmarks**. *Front Oncol* 2019, **9**:380.

6. Scott DA, Drake RR: **Glycosylation and its implications in breast cancer**. *Expert Rev Proteomics* 2019, **16**:665-680.

7. • Narimatsu Y, Joshi HJ, Nason R, Van Coillie J, Karlsson R, Sun L, Ye Z, Chen YH, Schjoldager KT, Steentoft C *et al.*: **An atlas of human glycosylation pathways enables display of the human glycome by gene engineered cells**. *Mol Cell* 2019, **75**:394-407.e5

This manuscript describes a technology based on isogenic Hek 293 cell libraries with combinatorially engineered glycosylation capacities designed to dissect functional aspects of glycosylation machinery. Cell based glycan arrays built based on the proposed strategy demonstrate binding of microbial adhesins to clustered patches of *O*-glycans organized by their presentation on proteins.

8. Rini JM, Esko JD *et al.*: **Glycosyltransferases and glycan-processing enzymes**. In *Essentials of Glycobiology.* Edited by Varki A, Cummings RD, Esko JD, Stanley P, Hart GW, Aebi M, Darvill AG, Kinoshita T, Packer NH. 2015:65-75 http://dx.doi.org/10.1101/glycobiology.3e.006.

9. Bennett EP, Mandel U, Clausen H, Gerken TA, Fritz TA, Tabak LA: **Control of mucin-type O-glycosylation: a classification of the polypeptide GalNAc-transferase gene family**. *Glycobiology* 2012, **22**:736-756.

10. Schjoldager KT, Joshi HJ, Kong Y, Goth CK, King SL, Wandall HH, Bennett EP, Vakhrushev SY, Clausen H: **Deconstruction of O-glycosylation—GalNAc-T isoforms direct distinct subsets of the O-glycoproteome**. *EMBO Rep* 2015, **16**:1713-1722.

11. Brockhausen I, Stanley P *et al.*: **O-GalNAc glycans**. In *Essentials of Glycobiology*. Edited by Varki A, Cummings RD, Esko JD, Stanley P, Hart GW, Aebi M, Darvill AG, Kinoshita T, Packer NH. 2015:113-123 http://dx.doi.org/10.1101/glycobiology.3e.010.

12. Yang Y, Franc V, Heck AJR: **Glycoproteomics: a balance between high-throughput and in-depth analysis**. *Trends Biotechnol* 2017, **35**:598-609.

13. Caval T, Heck AJR, Reiding KR: **Meta-heterogeneity: evaluating and describing the diversity in glycosylation between sites on the same glycoprotein**. *Mol Cell Proteomics* 2020 http://dx.doi.org/10.1074/mcp.R120.002093. ahead of print.

14. • Wilkinson H, Saldova R: **Current methods for the characterization of O-glycans**. *J Proteome Res* 2020, **19**:3890-3905

This review summarizes analytical methods for the analysis of the released *O*-glycans with a special emphasis how to address possible 'peeling' reactions during *O*-glycan extraction.

15. Wang C, Lu Y, Han J, Jin W, Li L, Zhang Y, Song X, Huang L, Wang Z: **Simultaneous release and labeling of O- and N-glycans allowing for rapid glycomic analysis by online LC-UV-ESI-MS/MS**. *J Proteome Res* 2018, **17**:2345-2357.

16. de Haan N, Yang S, Cipollo J, Wuhrer M: **Glycomics studies using sialic acid derivatization and mass spectrometry**. *Nat Rev Chem* 2020, **4**:229-242.

17. Smith J, Mittermayr S, Varadi C, Bones J: **Quantitative glycomics using liquid phase separations coupled to mass spectrometry**. *Analyst* 2017, **142**:700-720.

18. Keser T, Pavic T, Lauc G, Gornik O: **Comparison of 2-aminobenzamide, procainamide and RapiFluor-MS as derivatizing agents for high-throughput HILIC-UPLC-FLR-MS N-glycan analysis**. *Front Chem* 2018, **6**:324.

19. Hinneburg H, Schirmeister F, Korac P, Kolarich D: **N- and O-glycomics from minor amounts of formalin-fixed, paraffin-embedded tissue samples**. *Methods Mol Biol* 2017, **1503**:131-145.

20. Ashwood C, Lin CH, Thaysen-Andersen M, Packer NH: **Discrimination of isomers of released N- and O-glycans using diagnostic product ions in negative ion PGC-LC-ESI-MS/MS**. *J Am Soc Mass Spectrom* 2018, **29**:1194-1209.

21. Zhang T, Madunic K, Holst S, Zhang J, Jin C, Ten Dijke P, Karlsson NG, Stavenhagen K, Wuhrer M: **Development of a 96-well plate sample preparation method for integrated N- and O-glycomics using porous graphitized carbon liquid chromatography-mass spectrometry**. *Mol Omics* 2020, **16**:355-363.

22. Cho BG, Peng W, Mechref Y: **Separation of permethylated O-glycans, free oligosaccharides, and glycosphingolipid-glycans using porous graphitized carbon (PGC) column**. *Metabolites* 2020, **10**.

23. Gizaw ST, Gaunitz S, Novotny MV: **Highly sensitive O-glycan profiling for human serum proteins reveals gender-dependent changes in colorectal cancer patients**. *Anal Chem* 2019, **91**:6180-6189.

24. Kotsias M, Kozak RP, Gardner RA, Wuhrer M, Spencer DIR: **Improved and semi-automated reductive beta-elimination workflow for higher throughput protein O-glycosylation analysis**. *PLoS One* 2019, **14**:e0210759.

25. Moginger U, Grunewald S, Hennig R, Kuo CW, Schirmeister F, Voth H, Rapp E, Khoo KH, Seeberger PH, Simon JC *et al.*: **Alterations of the human skin N- and O-glycome in basal cell carcinoma and squamous cell carcinoma**. *Front Oncol* 2018, **8**:70.

26. Madunic K, Zhang T, Mayboroda OA, Holst S, Stavenhagen K, Jin C, Karlsson NG, Lageveen-Kammeijer GSM, Wuhrer M: **Colorectal cancer cell lines show striking diversity of their O-glycome reflecting the cellular differentiation phenotype**. *Cell Mol Life Sci* 2020 http://dx.doi.org/10.1007/s00018-020-03504-z. ahead of print.

27. Adamczyk B, Jin C, Polom K, Munoz P, Rojas-Macias MA, Zeeberg D, Boren M, Roviello F, Karlsson NG: **Sample handling of gastric tissue and O-glycan alterations in paired gastric cancer and non-tumorigenic tissues**. *Sci Rep* 2018, **8**:242.

28. Millan Martin S, Delporte C, Farrell A, Navas Iglesias N, McLoughlin N, Bones J: **Comparative analysis of monoclonal antibody N-glycosylation using stable isotope labelling and UPLC-fluorescence-MS**. *Analyst* 2015, **140**:1442-1447.

29. Kalmar JG, Butler KE, Baker ES, Muddiman DC: **Enhanced protocol for quantitative N-linked glycomics analysis using Individuality Normalization when Labeling with Isotopic Glycan Hydrazide Tags (INLIGHT)**. *Anal Bioanal Chem* 2020, **412**:7569-7579.

30. Afiuni-Zadeh S, Rogers JC, Snovida SI, Bomgarden RD, Griffin TJ: **AminoxyTMT: a novel multi-functional reagent for characterization of protein carbonylation**. *Biotechniques* 2016, **60**:186-188 190, 192–186.

31. Fujitani N, Furukawa J, Araki K, Fujioka T, Takegawa Y, Piao J, Nishioka T, Tamura T, Nikaido T, Ito M *et al.*: **Total cellular glycomics allows characterizing cells and streamlining the discovery process for cellular biomarkers**. *Proc Natl Acad Sci U S A* 2013, **110**:2105-2110.

32. Kameyama A, Thet Tin WW, Toyoda M, Sakaguchi M: **A practical method of liberating O-linked glycans from glycoproteins using hydroxylamine and an organic superbase**. *Biochem Biophys Res Commun* 2019, **513**:186-192.

33. • Kudelka MR, Nairn AV, Sardar MY, Sun X, Chaikof EL, Ju T, Moremen KW, Cummings RD: **Isotopic labeling with cellular O-glycome reporter/amplification (ICORA) for comparative O-glycomics of cultured cells**. *Glycobiology* 2018, **28**:214-222

Continuation of the CORA method for the *O*-glycan amplification for comparative *O*-glycmics study of cultured cells. During the *O*-glycome reporter amplification, cells were incubated with different GalNAcs with a 7 Da mass difference. This approach is a useful tool to compare *O*-glycome dynamics in health and disease.

34. Yu A, Zhao J, Peng W, Banazadeh A, Williamson SD, Goli M, Huang Y, Mechref Y: **Advances in mass spectrometry-based glycoproteomics**. *Electrophoresis* 2018, **39**:3104-3122.

35. Suttapitugsakul S, Sun F, Wu R: **Recent advances in glycoproteomic analysis by mass spectrometry**. *Anal Chem* 2020, **92**:267-291.

36. • Ruhaak LR, Xu G, Li Q, Goonatilleke E, Lebrilla CB: **Mass spectrometry approaches to glycomic and glycoproteomic analyses**. *Chem Rev* 2018, **118**:7886-7930

A recent update focusing on the role of mass spectrometry and chromatography for glycomics and glycoproteomics. This review could be useful for those who aim to join the field.

37. •• Khoo KH: **Advances toward mapping the full extent of protein site-specific O-GalNAc glycosylation that better reflects underlying glycomic complexity**. *Curr Opin Struct Biol* 2019, **56**:146-154

Another updated overview on how to address *O*-GalNAc glycosylation identification depth. Summarizes current analytical strategies, discusses advantages and disadvantages.

38. Medzihradszky KF, Kaasik K, Chalkley RJ: **Tissue-specific glycosylation at the glycopeptide level**. *Mol Cell Proteomics* 2015, **14**:2103-2110.

39. Stavenhagen K, Kayili HM, Holst S, Koeleman CAM, Engel R, Wouters D, Zeerleder S, Salih B, Wuhrer M: **N- and O-glycosylation analysis of human C1-inhibitor reveals extensive mucin-type O-glycosylation**. *Mol Cell Proteomics* 2018, **17**:1225-1238.

40. Momcilovic A, de Haan N, Hipgrave Ederveen AL, Bondt A, Koeleman CAM, Falck D, de Neef LA, Mesker WE, Tollenaar R, de Ru A *et al.*: **Simultaneous immunoglobulin A and G**

glycopeptide profiling for high-throughput applications. *Anal Chem* 2020, **92**:4518-4526.

41. Steentoft C, Vakhrushev SY, Joshi HJ, Kong Y, Vester-Christensen MB, Schjoldager KT, Lavrsen K, Dabelsteen S, Pedersen NB, Marcos-Silva L *et al.*: **Precision mapping of the human O-GalNAc glycoproteome through SimpleCell technology**. *EMBO J* 2013, **32**:1478-1488.

42. Riley NM, Bertozzi CR, Pitteri SJ: **A pragmatic guide to enrichment strategies for mass spectrometry-based glycoproteomics**. *Mol Cell Proteomics* 2020 http://dx.doi.org/10.1074/mcp.R120.002277. ahead of print.

43. Levery SB, Steentoft C, Halim A, Narimatsu Y, Clausen H, Vakhrushev SY: **Advances in mass spectrometry driven O-glycoproteomics**. *Biochim Biophys Acta* 2015, **1850**:33-42.

44. • Narimatsu Y, Joshi HJ, Schjoldager KT, Hintze J, Halim A, Steentoft C, Nason R, Mandel U, Bennett EP, Clausen H *et al.*: **Exploring regulation of protein O-glycosylation in isogenic human HEK293 cells by differential O-glycoproteomics**. *Mol Cell Proteomics* 2019, **18**:1396-1409

O-GalNAc differential glycoproteomic approach to address glycosylation occupancy on the macro-heterogeneity level. Several GalNac-transferase (GALNT1, T2, T3, T7, T10 or T11) knockouts were produced in isogenic Hek293 cell lines to explore the role of each enzyme in *O*-GalNAc glycosylation. This demonstrated that GALNT11 has a highly restricted function and serves low-density lipoprotein-related receptors in the linker region (C(6)XXXTC(1) between the ligand-binding repeats.

45. Tian E, Wang S, Zhang L, Zhang Y, Malicdan MC, Mao Y, Christoffersen C, Tabak LA, Schjoldager KT, Ten Hagen KG: **Galnt11 regulates kidney function by glycosylating the endocytosis receptor megalin to modulate ligand binding**. *Proc Natl Acad Sci U S A* 2019, **116**:25196-25202.

46. • Schumann B, Malaker SA, Wisnovsky SP, Debets MF, Agbay AJ, Fernandez D, Wagner LJS, Lin L, Li Z, Choi J *et al.*: **Bump-and-hole engineering identifies specific substrates of glycosyltransferases in living cells**. *Mol Cell* 2020, **78**:824-834.e15

Another approach for differential *O*-GalNAc glycoproteomics that used the engineering of living cells to tag glycans with editable chemical functionalities. A non-natural substrate is introduced into the biosynthetic pathway and engineered glycosyltransferases were used to incorporate chemically tagged sugars into the cell surface glycome of the living cell. This strategy was applied to study functional aspects of human polypeptide *N*-acetylgalactosaminyl transferases.

47. Choi J, Wagner LJS, Timmermans S, Malaker SA, Schumann B, Gray MA, Debets MF, Takashima M, Gehring J, Bertozzi CR: **Engineering orthogonal polypeptide GalNAc-transferase and UDP-sugar pairs**. *J Am Chem Soc* 2019, **141**:13442-13453.

48. •• Ye Z, Mao Y, Clausen H, Vakhrushev SY: **Glyco-DIA: a method for quantitative O-glycoproteomics with in silico-boosted glycopeptide libraries**. *Nat Methods* 2019, **16**:902-910

A novel GlycoDIA strategy for quantitative *O*-GalNAc glycoproteomics based on data independent acquisition (DIA). Using an *in silico* reconstruction of a human serum DIA library, 269 distinct *O*-GalNAc glycopeptides with up to 5 different Core 1 *O*-glycan structures were identified. This approach enables direct glycoproteomic analysis of small volumes of biofluids in a single shot analysis and requires no glycopeptide enrichment.

49. Caval T, Tian W, Yang Z, Clausen H, Heck AJR: **Direct quality control of glycoengineered erythropoietin variants**. *Nat Commun* 2018, **9**:3342.

50. • Wohlschlager T, Scheffler K, Forstenlehner IC, Skala W, Senn S, Damoc E, Holzmann J, Huber CG: **Native mass spectrometry combined with enzymatic dissection unravels glycoform heterogeneity of biopharmaceuticals**. *Nat Commun* 2018, **9**:1713

The authors applied high resolution native MS to characterize the glycosylation patter of Etanercept, a highly modified fusion protein containing 4 *N*-glycosites and up to 26 *O*-glycosites.

51. • Yen H-Y, Liko I, Gault J, Wu D, Struwe WB, Robinson CV: **Correlating glycoforms of DC-SIGN with stability using a combination of enzymatic digestion and ion mobility MS**. *Angew Chem (International Edition)* 2020, **59**:15560-15564

Combination of ion mobility MS and enzymatic treatment allowed for characterization of DC-SIGN *O*-glycosylation and revealed that the stability of the protein depends on the number of the attached *O*-glycans rather than the mass or structure of individual glycans.