Capillary Electrophoresis-Based N-Glycosylation Analysis in the Biomedical and Biopharmaceutical Fields

Renata Kun, Eszter Jóna, and Andras Guttman

Abstract

Glycomics has a growing interest in the biopharmaceutical industry and biomedical research requiring new high-performance and high-sensitivity bioanalytical tools. Analysis of N-glycosylation is very important during the development of protein therapeutics and it also plays a key role in biomarker discovery. The most frequently used glycoanalytical methods are capillary electrophoresis, liquid chromatography, and mass spectrometry. In this chapter, the capillary electrophoresis-based N-linked carbohydrate analysis methods are conferred with emphasis on its use in the biopharmaceutical and biomedical fields.

Keywords

Capillary electrophoresis · N-glycans · Biopharmaceuticals · Biomarkers

R. Kun
Horváth Csaba Memorial Laboratory of Bioseparation Sciences, Doctoral School of Molecular Medicine, Research Center for Molecular Medicine, Faculty of Medicine, University of Debrecen, Debrecen, Hungary

E. Jóna
Research Institute of Biomolecular and Chemical Engineering, University of Pannonia, Veszprem, Hungary

A. Guttman (✉)
Horváth Csaba Memorial Laboratory of Bioseparation Sciences, Doctoral School of Molecular Medicine, Research Center for Molecular Medicine, Faculty of Medicine, University of Debrecen, Debrecen, Hungary

Research Institute of Biomolecular and Chemical Engineering, University of Pannonia, Veszprem, Hungary

Abbreviations

AFP	alpha-fetoprotein
APTS	8-aminopyrene-1,3,6-trisulfonate
BHK	baby hamster kidney
CA	carbohydrate antigen
CEA	carcinoembryonic antigen
CE-LIF	capillary electrophoresis with laser-induced fluorescence detection
CHO	Chinese hamster ovary
HILIC	hydrophilic interaction liquid chromatography
HPLC	high-performance liquid chromatography
mAB	monoclonal antibody
MS	mass spectrometry
NS0	murine myeloma cell line
PNGase F	peptide-N-glycosidase F
PSA	prostate-specific antigen

A. V. Colnaghi Simionato (ed.), *Separation Techniques Applied to Omics Sciences*, Advances in Experimental Medicine and Biology 1336, https://doi.org/10.1007/978-3-030-77252-9_7

1 Introduction

Glycosylation of proteins is one of the most important post-translation modifications, which impacts their function and their lifespan, also taking part in important biochemical and physiological processes as well as in cell-cell interactions. The buildup of the glycan structure may therefore modify the cell functions and can serve as indicators of various diseases. In the course of N-glycosylation, the carbohydrate structures bind to the polypeptide chains of the proteins being synthesized (co-translational) and then modified post-translationally [1, 2]. The glycan structures are usually made up of several glycoforms, which significantly increase the structural diversity of glycans. This so-called microheterogeneity depends on the expression, concentration, and kinetic features of glycosyltransferases and glycosidases. The glycoforms might have various binding sites within a protein, which is referred to as glycosylation macroheterogeneity (site specificity) [3–6].

The structural diversity of recombinant glycoproteins is very important for the pharmaceutical industry to avoid unwanted side effects and allergic reactions. During the development of these new-generation medicines, the producing microorganism must be chosen carefully to avoid immunogenic effects caused by nonhuman glycan epitopes. The most immunogenic nonhuman sugar residues are alpha-1,3-galactosylation and N-glycolylneuraminic acid (Neu5Gc) [7–9].

2 The Biochemical Background of Glycosylation

Asparagine (N)-linked glycans have three main structural subtypes. If it contains only mannose in addition to the core structure (Fig. 1, left panel), it is called “high mannose” type (Fig. 1, right panel). Hybrid glycans consist of both mannose and other sugar units in addition to the core. Complex-type glycans have other (non-mannose) sugar units added to the core structure (Fig. 1, right panel) [3, 10].

Another major type of protein glycosylation (not discussed in this chapter) is O-glycosylation via Thr or Ser residues. O-Glycans are synthetized in the Golgi apparatus and they predominantly appear on the surface of cells synthetizing mucins and on epithelial cell surfaces rich in serine and threonine [2, 11–15].

3 Glycobiomarker Discovery

Modifications in N-glycan structures significantly influence the half-life of proteins, their maturity state, cellular adhesion characteristics, migration, tumor invasion, and the formation of metastases. Various serological assays are available to identify organ-specific tumor glycobiomarkers, and they provide information on the prognosis of the disease [8, 16–18]. Biomarker assays recognize the glycan structures on the surface of the cells, for example, carbohydrate antigen (CA) 19-9 (pancreas/colorectal/gastro-carbohydrate antigen), CA 72-4 (colorectal/gastric), CA 125 (ovary), CA 15-3 (breast), AFP-L3 (hepatic cells), and PSA (prostate-specific antigen). Carcinoembryonic antigen (CEA) is a general and diagnostically widespread tumor marker, rich in N-glycan structures. The serum level of these biomarkers can be specific for determination of tumor genesis; therefore, the mapping of new, more specific glycobiomarkers is desirable in the future (Fig. 2) [16–25].

4 Glycosylated Biopharmaceuticals

The asparagine-linked carbohydrate moieties also have high significance in the pharmaceutical industry, because most of the new-generation biotherapeutics are glycosylated protein-based medicines, manufactured by recombinant techniques. In addition to monoclonal antibody (mAb)-type drugs, a number of hormones, coagulation factors, and lecithin-type compounds are continuously entering the market these days. Modifications of their linked glycan structures

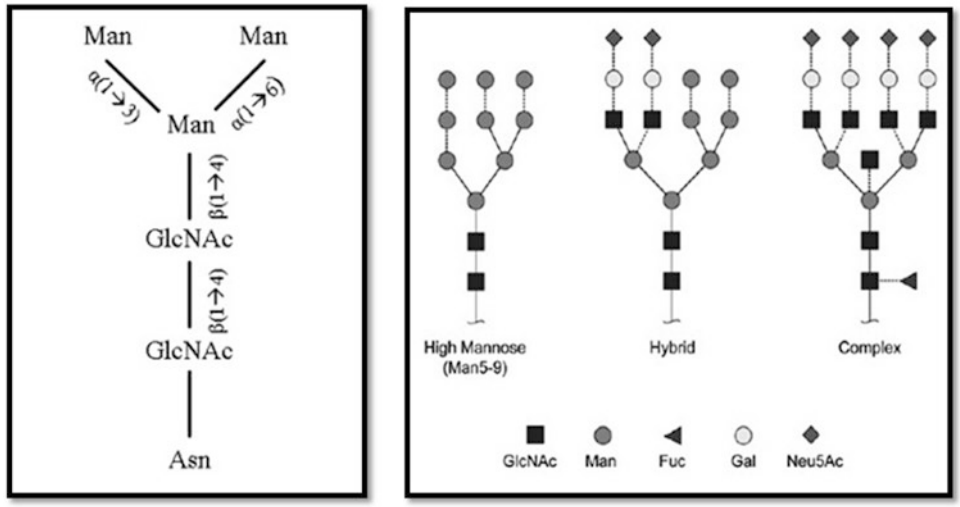

Fig. 1 The trimannosyl core structure of N-linked glycans (left panel) and the main N-glycan structure subtypes (right panel). Symbols: ■ N-Acetylglucosamine; ● Mannose; ◄ Fucose; ○ Galactose; ♦ N-acetylneuraminic acid. With permissions from [2, 10]

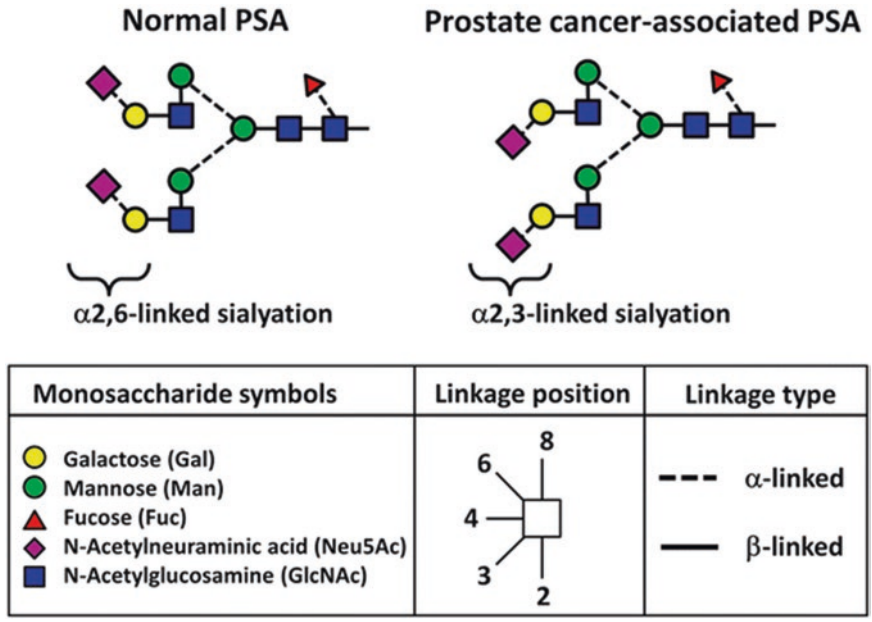

Fig. 2 Differences between normal and prostate cancer patients in the N-glycosylation of prostate-specific antigen (PSA). The terminal sialic acids are α2,3 linked on the aberrant PSA. (With permission from [21])

highly influence their efficiency, stability, safety, and half-life (Fig. 3) [6, 7, 26–29].

The glycosylation decoration of biological medicines greatly depends on the type of the producing microorganism/mammalian cell lines and the production environment. Nonhuman cell lines and microorganisms may synthesize immunogenic glycan residues such as N-glycolylneuraminic acid (Neu5Gc) (CHO cell line), alpha1,3-galactose epitope (BHK, NS0 cells), core alpha1,3-fucose (insects), beta1,2-xylose/core and alpha1,3-fucose (plants), and

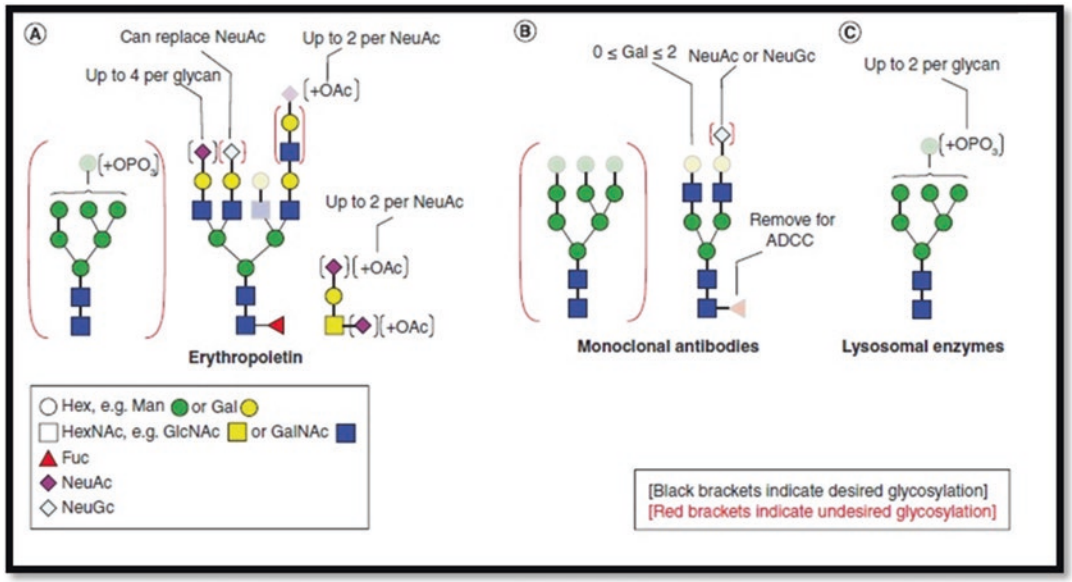

Fig. 3 Glycosylation of biopharmaceuticals. The red brackets indicate undesired/immunogenic sugar epitopes on glycopeptides from nonhuman expression systems. (With permission from [28])

hyper-mannosylation (yeast) [3, 7, 10, 30]. The abovementioned sugar monomers are immunogenic, so minimizing their presence by better optimization of the production conditions (glycoengineering) is very important. The decrease and avoidance of extreme microheterogeneities in production cells facilitated more reproducible manufacturing of both innovative and biosimilar medicines [3, 5, 7].

5 Glycan Analysis Options

Analysis of the asparagine-linked carbohydrate moieties of glycoproteins is very important for the pharmaceutical industry and in the area of biomarker research requiring high sensitivity and high-resolution separation and detection methods. These bioanalytical techniques should provide detailed N-glycan profile information, including data about linkages and positional isomers. High-sensitivity glycoanalytical tools are readily available today on a wide scale to map protein glycosylations, help to identify smaller structural dissimilarities in the carbohydrate structures, and discover new glycan epitopes [6, 31–33]. The most frequently utilized methods are capillary electrophoresis (CE), high-performance liquid chromatography (HPLC), hydrophilic interaction liquid chromatography (HILIC), affinity chromatography, mass spectrometry (MS), and their combinations. Most of these methods require removal of the N-linked oligosaccharides from the glycoproteins by endoglycosidase enzymes, such as peptide-N-glycosidase F (PNGase F), followed by labeling, e.g., with fluorescent dyes. Glycans can later be digested with exoglycosidase enzymes for sequence (residue, linkage, and positional) analysis (Fig. 4) [33–36].

HPLC and UPLC are widely used methods in the profile analysis of released glycans. Combined with mass spectrometry, it provides some structural information about the glycans [37, 38]. An excellent method for the N-glycosylation analysis of intact glycoproteins is MS combined with RP-HPLC, providing detailed information about the heterogeneity of carbohydrate binding sites, most frequently combined with ESI-TOF analyzer. MALDI is another efficient way for glycan analysis, but its accuracy is not as good as with HPLC. However, it is but appropriate for O-glycan

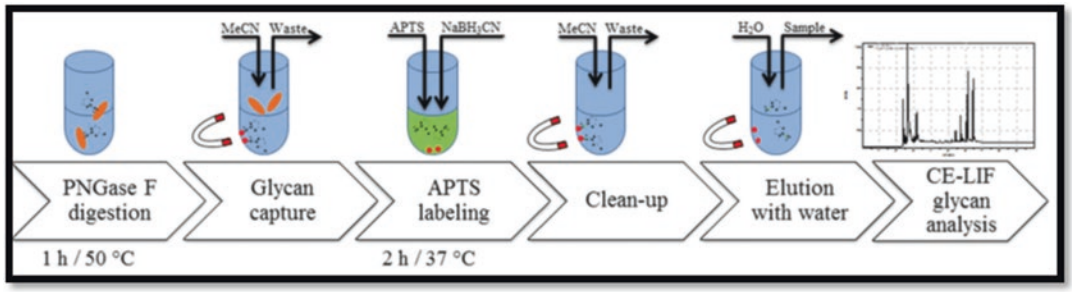

Fig. 4 Glycosylation analysis workflow including endoglycosidase (PNGase F) digestion, capturing the released glycans by magnetic beads, fluorophore labeling of the carbohydrates on the beads, cleanup, and CE-LIF analysis after elution. (With permission from [33])

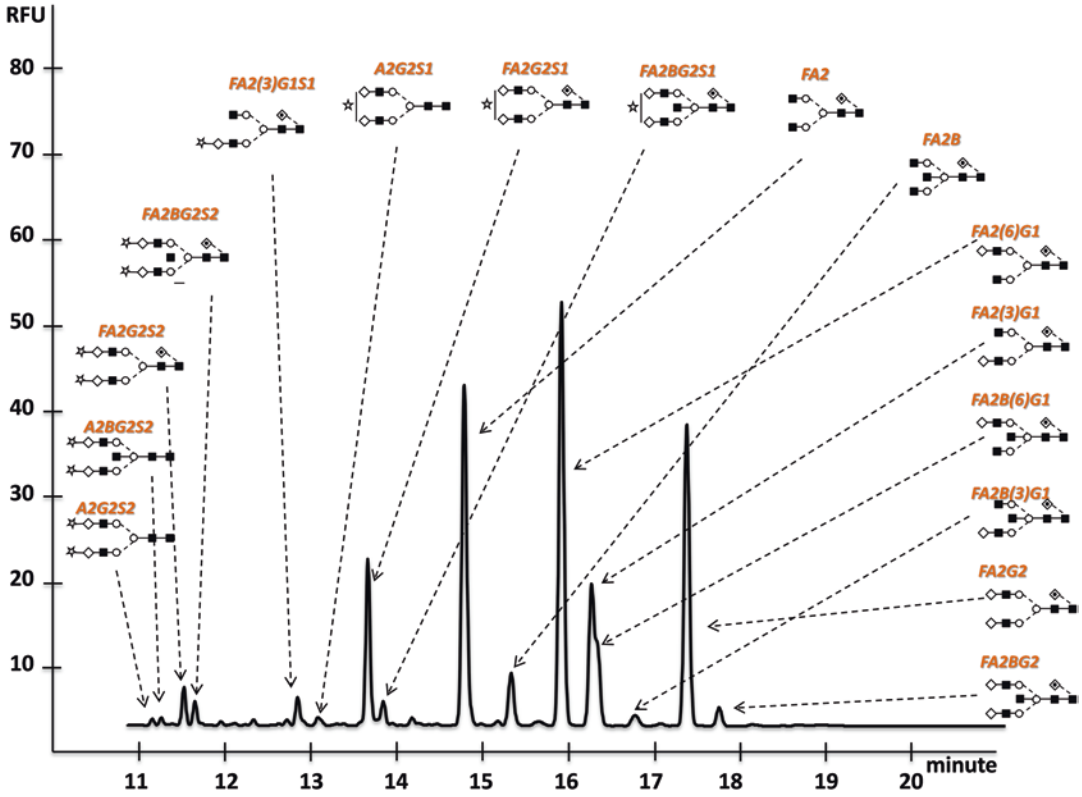

Fig. 5 Capillary electrophoresis analysis of endoglycosidase-released and 8-aminopyrene-1,3,6-trisulfontate-labeled human IgG N-glycans using laser-induced fluorescence detection

analysis/characterization [39–43]. The advent of HILIC chromatography in carbohydrate analysis was an important step forward, as this method is capable of efficiently separating N-glycans [34, 44].

Capillary electrophoresis (CE) has proven to be one of the most excellent separation methods for the analysis of complex N-glycan structures (Fig. 5). Coupled with laser-induced fluorescence detection (LIF), it is possible to reach very high sensitivity and resolution. In the case of carbohydrate sequencing, serial exoglycosidase digestion is required, similar to that of LC. The fluorescent labeling of glycans for CE-LIF is performed

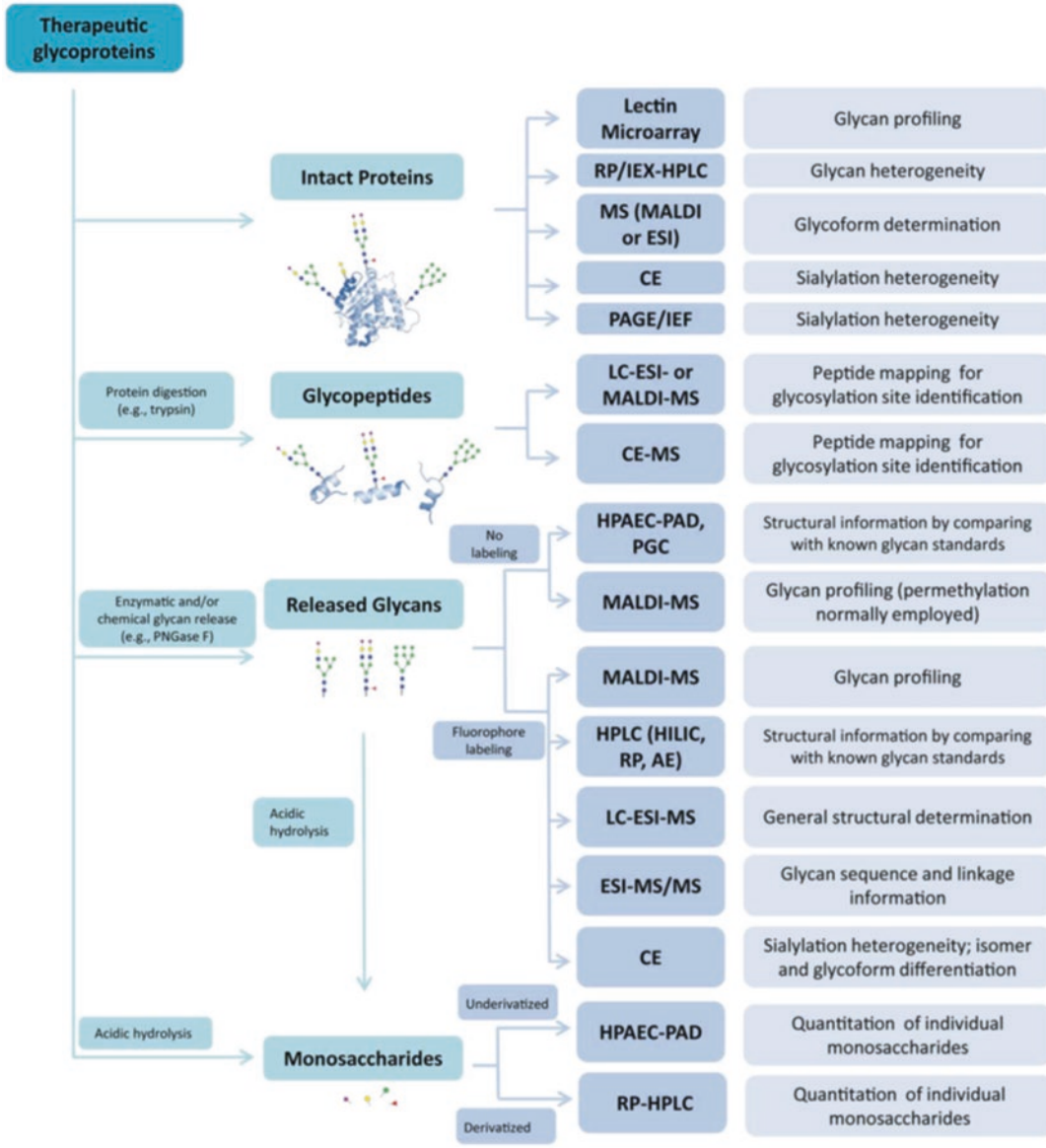

Fig. 6 Summary of common glycosylation analysis workflows and the main groups of the associated analytical applications. (With permission from [60])

using APTS (8-aminopyrene-1,3,6-trisulfonate). Various subsets of CE such as micellar electrokinetic chromatography or isoelectric focusing increase the efficiency and resolution of the separation at the glycopeptide and/or glycoprotein level with lower sample requirement than that of MS or HPLC [45–48].

These commonly used analytical methods can give valuable structural information about the main carbohydrate groups on glycopeptides, intact proteins in addition to released glycans and monosaccharides. Intact protein glycan mapping is possible with lectin microarrays and CE or MS [43, 46, 49, 50]. MS coupled with ESI or MALDI can determine various glycoforms. CE with MS can reveal sialylation heterogeneity of intact proteins and can give detailed site identification of glycopeptides as well [41, 51, 52]. Lectin micro-

arrays can detect glycan-lectin interactions of intact proteins and give useful information about glycoconjugates, but cannot provide structural information [53]. MALDI-MS offers detailed structural information about intact glycoproteins including linkage and branches and can provide information about glycosylation site specificity, but with lower mass accuracy [54]. Charge-based electrophoresis such as capillary isoelectrofocusing (cIEF) is useful for quality control testing for sialylated species [55]. HPLC with ESI-MS or with MALDI-MS is widely used to gain information about protein glycosylation sites and occupancy also suitable for rapid glycopeptide profiling [56, 57]. RPLC-MS could provide complete sequence analysis of glycoproteins but needs higher sample amounts. On the other hand, CE in combination with MS offers high-resolution analysis of glycoproteins providing useful information about the entire glycan structures while only needing very low sample amounts [58, 59].

6 Summary

The growing interest in glycomics in the pharmaceutical industry and in biomedical research demanded the development of high-resolution and high-sensitivity glycoanalytical techniques. N-Glycosylation analysis is very important for the development of monoclonal antibody-based and new modality medicines, showing promising results in the field of biomarker research. Due to the complex structures of N-linked carbohydrates, their analysis is challenging that requires new-generation, high-resolution/high-sensitivity methods such as CE-LIF and various liquid chromatography-based methods preferably connected to mass spectrometry. Indeed, the additional mass spectrometry data provides deeper structural information, but mostly requires coupling to liquid phase separation methods as described in Fig. 6.

Acknowledgments The authors gratefully acknowledge the grants from the National Research, Development and Innovation Office (K116263, NN127062, 2018-2.1.17-TÉT-KR-2018-00010). This work was also sponsored by the BIONANO_GINOP-2.3.2-15-2016-00017 project of the Hungarian Government. This is contribution #161 from the Horváth Csaba Memorial Laboratory of Bioseparation Sciences.

References

1. Shental-Bechor D, Levy Y (2009) Folding of glycoproteins: toward understanding the biophysics of the glycosylation code. Curr Opin Struct Biol 19(5):524–533
2. Varki A et al (2015) Symbol nomenclature for graphical representations of Glycans. Glycobiology 25(12):1323–1324
3. Brooks SA (2006) Protein glycosylation in diverse cell systems: implications for modification and analysis of recombinant proteins. Expert Rev Proteomics 3(3):345–359
4. Kornfeld R, Kornfeld S (1985) Assembly of asparagine-linked oligosaccharides. Annu Rev Biochem 54:631–664
5. Marino K et al (2010) A systematic approach to protein glycosylation analysis: a path through the maze. Nat Chem Biol 6(10):713–723
6. Szekrenyes A et al (2015) Sample preparation for N-glycosylation analysis of therapeutic monoclonal antibodies by electrophoresis. Methods Mol Biol 1274:183–195
7. Dicker M, Strasser R (2015) Using glyco-engineering to produce therapeutic proteins. Expert Opin Biol Ther 15(10):1501–1516
8. Dube DH, Bertozzi CR (2005) Glycans in cancer and inflammation. Potential for therapeutics and diagnostics. Nat Rev Drug Discov 4(6):477–488
9. Shrimal S, Gilmore R (2013) Glycosylation of closely spaced acceptor sites in human glycoproteins. J Cell Sci 126(23):5513–5523
10. Brooks SA (2009) Strategies for analysis of the glycosylation of proteins: current status and future perspectives. Mol Biotechnol 43(1):76–88
11. Brockhausen I (1999) Pathways of O-glycan biosynthesis in cancer cells. Biochim Biophys Acta 1473(1):67–95
12. Hanisch FG (2001) O-glycosylation of the mucin type. Biol Chem 382(2):143–149
13. Ito Y et al (2005) Structural approaches to the study of oligosaccharides in glycoprotein quality control. Curr Opin Struct Biol 15(5):481–489
14. Spiro RG (1973) Glycoproteins. Adv Protein Chem 27:349–467
15. Spiro RG (2002) Protein glycosylation: nature, distribution, enzymatic formation, and disease implications of glycopeptide bonds. Glycobiology 12(4):43r–56r
16. Drake RR (2015) Glycosylation and cancer: moving glycomics to the forefront. Adv Cancer Res 126:1–10

17. Pearce OMT (2018) Cancer glycan epitopes: biosynthesis, structure and function. Glycobiology 28(9):670–696
18. Reis CA et al (2010) Alterations in glycosylation as biomarkers for cancer detection. J Clin Pathol 63(4):322–329
19. Gilgunn S et al (2013) Aberrant PSA glycosylation-a sweet predictor of prostate cancer. Nat Rev Urol 10(2):99–107
20. Goldstein MJ, Mitchell EP (2005) Carcinoembryonic antigen in the staging and follow-up of patients with colorectal cancer. Cancer Investig 23(4):338–351
21. Hatakeyama S et al (2017) Recent progress and perspectives on prostate cancer biomarkers. Int J Clin Oncol 22(2):214–221
22. Varki A, Cummings R, Esko J, et al., editors. (1999) Essentials of Glycobiology. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press. Chapter 35, Glycosylation Changes in Cancer.
23. Li M, Song L, Qin X (2010) Glycan changes: cancer metastasis and anti-cancer vaccines. J Biosci 35(4):665–673
24. Locker GY et al (2006) ASCO 2006 update of recommendations for the use of tumor markers in gastrointestinal cancer. J Clin Oncol 24(33):5313–5327
25. Pinho SS, Reis CA (2015) Glycosylation in cancer: mechanisms and clinical implications. Nat Rev Cancer 15(9):540–555
26. Brinks V et al (2011) Quality of original and biosimilar Epoetin products. Pharm Res 28(2):386–393
27. Lingg N et al (2012) The sweet tooth of biopharmaceuticals: importance of recombinant protein glycosylation analysis. Biotechnol J 7(12):1462–1472
28. Oh MJ et al (2016) Analytical detection and characterization of biopharmaceutical glycosylation by MS. Bioanalysis 8(7):711–727
29. Wacker C et al (2011) Glycosylation profiles of therapeutic antibody pharmaceuticals. Eur J Pharm Biopharm 79(3):503–507
30. Hoja-Lukowicz D et al (2013) L1CAM from human melanoma carries a novel type of N-glycan with Galbeta1-4Galbeta1- motif. Involvement of N-linked glycans in migratory and invasive behaviour of melanoma cells. Glycoconj J 30(3):205–225
31. Bielik AM, Zaia J (2010) Historical overview of glycoanalysis. Methods Mol Biol 600:9–30
32. Dimitrov DS, Marks JD (2009) Therapeutic antibodies: current state and future trends--is a paradigm change coming soon? Methods Mol Biol 525:1–27, xiii
33. Szigeti M et al (2016) Fully automated sample preparation for ultrafast N-glycosylation analysis of antibody therapeutics. J Lab Autom 21(2):281–286
34. Melmer M et al (2010) HILIC analysis of fluorescence-labeled N-glycans from recombinant biopharmaceuticals. Anal Bioanal Chem 398(2):905–914
35. Reusch D et al (2015) Comparison of methods for the analysis of therapeutic immunoglobulin G Fc-glycosylation profiles-part 2: mass spectrometric methods. MAbs 7(4):732–742
36. Vreeker GC, Wuhrer M (2017) Reversed-phase separation methods for glycan analysis. Anal Bioanal Chem 409(2):359–378
37. Veillon L et al (2017) Characterization of isomeric glycan structures by LC-MS/MS. Electrophoresis 38(17):2100–2114
38. Zhou S et al (2017) Direct comparison of derivatization strategies for LC-MS/MS analysis of N-glycans. Analyst 142(23):4446–4455
39. Dell A, Morris HR (2001) Glycoprotein structure determination by mass spectrometry. Science 291(5512):2351–2356
40. Geyer H, Geyer R (2006) Strategies for analysis of glycoprotein glycosylation. Biochim Biophys Acta 1764(12):1853–1869
41. Grünwald-Gruber C et al (2017) Determination of true ratios of different N-glycan structures in electrospray ionization mass spectrometry. Anal Bioanal Chem 409(10):2519–2530
42. Higel F et al (2013) Reversed-phase liquid-chromatographic mass spectrometric N-glycan analysis of biopharmaceuticals. Anal Bioanal Chem 405(8):2481–2493
43. Zhang Z, Pan H, Chen X (2009) Mass spectrometry for structural characterization of therapeutic antibodies. Mass Spectrom Rev 28(1):147–176
44. Ruhaak LR et al (2008) Hydrophilic interaction chromatography-based high-throughput sample preparation method for N-glycan analysis from total human plasma glycoproteins. Anal Chem 80(15):6119–6126
45. Guttman A (1996) High-resolution carbohydrate profiling by capillary gel electrophoresis. Nature 380(6573):461–462
46. Guttman A (2013) Capillary electrophoresis in the N-glycosylation analysis of biopharmaceuticals. Trac Trends Anal Chem 48:132–143
47. Kamoda S, Ishikawa R, Kakehi K (2006) Capillary electrophoresis with laser-induced fluorescence detection for detailed studies on N-linked oligosaccharide profile of therapeutic recombinant monoclonal antibodies. J Chromatogr A 1133(1–2):332–339
48. Ma S, Nashabeh W (1999) Carbohydrate analysis of a chimeric recombinant monoclonal antibody by capillary electrophoresis with laser-induced fluorescence detection. Anal Chem 71(22):5185–5192
49. North SJ et al (2009) Mass spectrometry in the analysis of N-linked and O-linked glycans. Curr Opin Struct Biol 19(5):498–506
50. Zhang L, Luo S, Zhang B (2016) The use of lectin microarray for assessing glycosylation of therapeutic proteins. MAbs 8(3):524–535
51. Gennaro LA, Salas-Solano O, Ma S (2006) Capillary electrophoresis-mass spectrometry as a characterization tool for therapeutic proteins. Anal Biochem 355(2):249–258
52. Nishikaze T (2017) Sensitive and structure-informative N-glycosylation analysis by MALDI-MS; ionization, fragmentation, and derivatization. Mass Spectrom (Tokyo, Jpn) 6(1):A0060–A0060

53. Hu S, Wong DT (2009) Lectin microarray. Proteomics Clin Appl 3(2):148–154
54. Mechref Y, Novotny MV, Krishnan C (2003) Structural characterization of oligosaccharides using MALDI-TOF/TOF tandem mass spectrometry. Anal Chem 75(18):4895–4903
55. Markely LR et al (2016) A high-throughput capillary isoelectric focusing immunoassay for fingerprinting protein sialylation. Biotechnol Prog 32(1):235–241
56. Gillmeister MP et al (2009) An HPLC-MALDI MS method for N-glycan analyses using smaller size samples: application to monitor glycan modulation by medium conditions. Glycoconj J 26(9):1135–1149
57. Han L, Costello CE (2013) Mass spectrometry of glycans. Biochem Biokhimiia 78(7):710–720
58. Pioch M, Bunz SC, Neususs C (2012) Capillary electrophoresis/mass spectrometry relevant to pharmaceutical and biotechnological applications. Electrophoresis 33(11):1517–1530
59. Zhou S, Hu Y, Mechref Y (2016) High-temperature LC-MS/MS of permethylated glycans derived from glycoproteins. Electrophoresis 37(11):1506–1513
60. Zhang L, Luo S, Zhang B (2016) Glycan analysis of therapeutic glycoproteins. MAbs 8(2):205–215