

Chapter 4

Capillary (Gel) Electrophoresis-Based Methods for Immunoglobulin (G) Glycosylation Analysis



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Abstract The in-depth characterization of protein glycosylation has become indispensable in many research fields and in the biopharmaceutical industry. Especially knowledge about modulations in immunoglobulin G (IgG) *N*-glycosylation and their effect on immunity enabled a better understanding of human diseases and the development of new, more effective drugs for their treatment. This chapter provides a deeper insight into capillary (gel) electrophoresis-based (C(G)E) glycan analysis, addressing its impressive performance and possibilities, its great potential regarding

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real high-throughput for large cohort studies, as well as its challenges and limitations. We focus on the latest developments with respect to miniaturization and mass spectrometry coupling, as well as data analysis and interpretation. The use of exoglycosidase sequencing in combination with current C(G)E technology is discussed, highlighting possible difficulties and pitfalls. The application section describes the detailed characterization of *N*-glycosylation, utilizing multiplexed CGE with laser-induced fluorescence detection (xCGE-LIF). Besides a comprehensive overview on antibody glycosylation by comparing species-specific IgGs and human immunoglobulins A, D, E, G, and M, the chapter comprises a comparison of therapeutic monoclonal antibodies from different production cell lines, as well as a detailed characterization of Fab and Fc glycosylation. These examples illustrate the full potential of C(G)E, resolving the smallest differences in sugar composition and structure.

Keywords Capillary gel electrophoresis · *N*-glycosylation · Biopharmaceuticals · Immunoglobulin · IgA · IgD · IgE · IgG · IgM · Monoclonal antibody · mAb · APTS · xCGE-LIF · CE-MS · Chip-CE · Exoglycosidase

Abbreviations

APTS	8-aminopyrene-1,3,6-trisulfonic-acid
CE	Capillary electrophoresis
CGE	Capillary gel electrophoresis
CHO	Chinese hamster ovary
CQA	Critical quality attribute
CZE	Capillary zone electrophoresis
DB	Database
EOF	Electroosmotic flow
ESI	Electrospray ionization
Fab	Fragment antigen binding
Fc	Fragment crystallizable
Fuc	Fucose
Gal	Galactose
GlcNAc	<i>N</i> -acetylglucosamine
GU	Glucose units
HAGR	Hepatic asialo-glycoprotein receptor
HILIC	Hydrophilic interaction liquid chromatography
HMOS	Human milk oligosaccharides
HPLC	High-performance liquid chromatography
Ig	Immunoglobulin
LIF	Laser-induced fluorescence detection
mAb	Monoclonal antibody
MALDI-TOF-MS	Matrix-assisted laser desorption ionization with time-of-flight MS

Man	Mannose
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MTU	Migration time unit
MTU''	Double aligned migration time units
Neu5Ac	<i>N</i> -acetyl-neuraminic acid
Neu5Gc	<i>N</i> -glycolyl-neuraminic acid
NMR	Nuclear magnetic resonance
Sia	Sialic acid
SNFG	Symbol nomenclature for glycans
UPLC	Ultra-performance liquid chromatography
xCGE-LIF	Multiplexed capillary gel electrophoresis with laser-induced fluorescence detection

4.1 Historical Background

Electrophoresis was born more than 200 years ago (Ruess 1809), even long before the concept of chromatography was described. However, it took about 150 more years before the use of capillaries was introduced (Hjertén 1967). From the late 1960s, it took an additional decade to establish capillary electrophoresis (*CE*) as it is most widely known today, enabling separations that seemed unattainable at that time (Jorgenson and DeArman Lukacs 1981; Jorgenson and Lukacs 1981a, b). The increased efficiency and the amazing separation capabilities together with short analysis times, induced a growing interest among the scientific community. Since then, *CE* has constantly been improved and has become an important tool in the analysis of a wide class of compounds, from small ions over amino acids and peptides/proteins to large DNA fragments (Saraswathy and Ramalingam 2011). However, the employment of *CE* for glycan analysis lagged behind other commonly used analytical techniques. Attempts to use *CE* for glycan analysis started in the 1990s, but they were rather humble and not broadly embraced—neither by the glycoscientific community nor by industrial laboratories. Today, 40 years since the potential of *CE* was recognized, the advantages of the method for analysis of glycans are becoming increasingly obvious and appreciated. Ready-to-use *CE* methods and kits on the market (glyXera 2020; Thermo Fisher Scientific 2018; Ludger 2018; ProZyme 2018; SCIEX 2018; PerkinElmer 2018) enable fast and robust comparison of glycan profiles, which makes the technology more routine in academic research and industry. In the following sections, we will emphasize some of the reasons why *CE* is gaining popularity and underline why this technology is attractive for the biopharmaceutical industry, especially regarding the analysis of immunoglobulin (*Ig*) glycosylation. Additionally, some current and future challenges, as well as possible future methodological developments, will be addressed.

4.2 Background: Principles of Capillary (Gel) Electrophoresis (C(G)E)

Capillary electrophoresis is the umbrella term for various capillary electrophoretic separation techniques and methods, such as capillary zone electrophoresis (*CZE*) and capillary gel electrophoresis (*CGE*). The differential migration of charged molecular species (ions) in a narrow capillary (25–75 μm) containing an electrolyte solution under the influence of high voltage (usually 10–30 kV, generating an electric field) is the basis for separation by CE (Watson 2012). An analyte is attracted to either the anode (positive electrode) or the cathode (negative electrode), and its movement (electrophoretic mobility) in an electric field results from both electrical and frictional force contributions. The movement of a charged analyte through a conductive solution is dependent on the charge of the analyte and the magnitude of the applied electric field. Additionally, a frictional force will impede the movement induced by the electrical field to a certain extent, dependent on the viscosity of the electrolyte solution and the analyte radius/molecular size or shape. Thus, the mobility and, therefore, the separation is based on the size-to-charge ratio of the analytes. Besides electrophoretic velocity, electroosmotic flow (*EOF*) is influencing the movement of the analyte through the capillary. EOF is the bulk liquid motion in a capillary when a high voltage (electric field) is applied. In a capillary composed of fused silica, the surface possesses negative charges (SiO^-) over a wide pH range (pH 4–12). This negatively charged surface attracts positively charged electrolytes from the running buffer. Along an electric field, these cations (enriched at the capillary wall) move towards the cathode. Due to solvation and liquid viscosity, these cations drag the surrounded bulk solvent with them along the capillary axis, creating a net flow toward the cathode, the EOF. Thus, all analytes, irrespective of their charge, are pushed in one direction, enabling simultaneous separation and detection of cationic (EOF + ion migration = fastest), neutral (EOF = middle) and anionic (EOF – ion migration = slowest) analytes (Holland et al. 1997). Although this is rather a rough summary of the separation mechanism, the reader should recognize that the parameters involved in optimizing the technique to produce separation are very complex. However, the effort pays off as very small changes in molecular structure can lead to quite marked differences in migration if variables are carefully balanced. There is a large volume of literature regarding CE separations of glycans exploiting a variety of possible setups, methods, modes, and parameters (e.g., capillary material, dimensions, and surface coating, voltage, current, polarity, temperature, buffer type and concentration, pH, additives/modifiers, injection technique, detection mode, etc.) that is beyond the scope of this review (Campa and Rossi 2008; Ruhaak et al. 2010a; Lu et al. 2018). However, due to a boom of applications in the field of glycan analysis and particular interest in the biopharmaceutical industry, one technique deserves to be further dissected—capillary gel electrophoresis (CGE).

After providing the complete genetic blueprint of human life (Collins et al. 2004), CGE-based Genetic Analyzers (DNA analyzers) found their application in the field of glycan analysis, with a more complex challenge but a new hope of deciphering the

glycome (Schwarzer et al. 2008; Laroy et al. 2006; Callewaert et al. 2001; Ruhaak et al. 2010b). These instruments combine four great advantageous features, which make them very powerful analytical tools in glycoscience. First, in CGE, the abovementioned EOF is typically completely suppressed by employing a neutral capillary (permanently or dynamically coated) filled with a gel mesh polymer. The use of such a gel buffer increases the viscosity of the electrophoresis medium. Consequently, due to the role of viscosity in frictional drag, this increases the time analytes spend in the capillary (decrease of electrophoretic mobility), while decreasing diffusion and thereby improving the separation (Watson 2012; Guttman et al. 1994; Luo et al. 2010). Thanks to this first advantageous feature, glycans are separated based on mass-to-charge ratio and molecular size/shape (hydrodynamic volumes) with high resolution (Guttman et al. 1996a; Guttman and Herrick 1996). Second, the large surface-to-volume ratio of narrow capillaries enables a very good heat transfer. This in turn raises the possibility to apply very high electric fields, which increases the electrophoretic mobility and consequently decreases the analysis time. Third, besides its high separation efficiency in a short time, this method is also attractive due to the possibility to employ laser-induced fluorescence (*LIF*) detection, known for its impressive sensitivity (low attomole range) (Guttman 1996; Hennig et al. 2011a). The fourth advantage is the possibility to employ a multi-capillary format (multiplexing), incorporating up to 96 capillaries in parallel, so that hundreds of samples can be measured by CGE per day (Mittermayr et al. 2013). As a result, modern multiplexed capillary gel electrophoresis with laser-induced fluorescence detection (*xCGE-LIF*) instruments offer an amazing high-throughput, separation performance and sensitivity. Another attractive option provided, e.g., by Applied Biosystems Genetic Analyzers, is a recording of up to six different fluorescent dyes (in six independent detection traces/channels) simultaneously within one run (all excited by only one laser). As described later, this special feature has been exploited for internal alignment of migration times (glycans and internal base pair standard labeled with different dyes), giving long-time stable migration times (Callewaert et al. 2001; Laroy et al. 2006; Schwarzer et al. 2008; Reusch et al. 2014). The basic setup of a Genetic Analyzer ((x)CGE-LIF system) and the principle of glycan separation is depicted in Fig. 4.1.

The majority of glycans are uncharged and therefore would neither be electrokinetically injected nor migrate in an electric field when EOF is suppressed. Additionally, glycans lack a chromophore/fluorophore necessary for optical detection (Guttman et al. 1996b). Thus, an ideal label for C(G)E-LIF needs to have high fluorescent yield, carry (sufficient) negative charges stable over a broad pH range (for electric field-mediated mobilization), and it needs to have an excitation wavelength corresponding to the output wavelength of commercially available lasers (commonly argon-ion laser) (Briggs et al. 2009). All these requirements are met in 8-aminopyrene-1,3,6-trisulfonic-acid (*APTS*) fluorescent dye (Evangelista et al. 1995; Ruhaak et al. 2010b), which is almost universally employed in CE-based glycan analysis today. High absorptivity and quantum yield of *APTS* enable a highly sensitive LIF detection, thus making CE an appropriate method for high-sensitivity glycan analysis, even with low sample amounts (Guttman et al. 1996a).

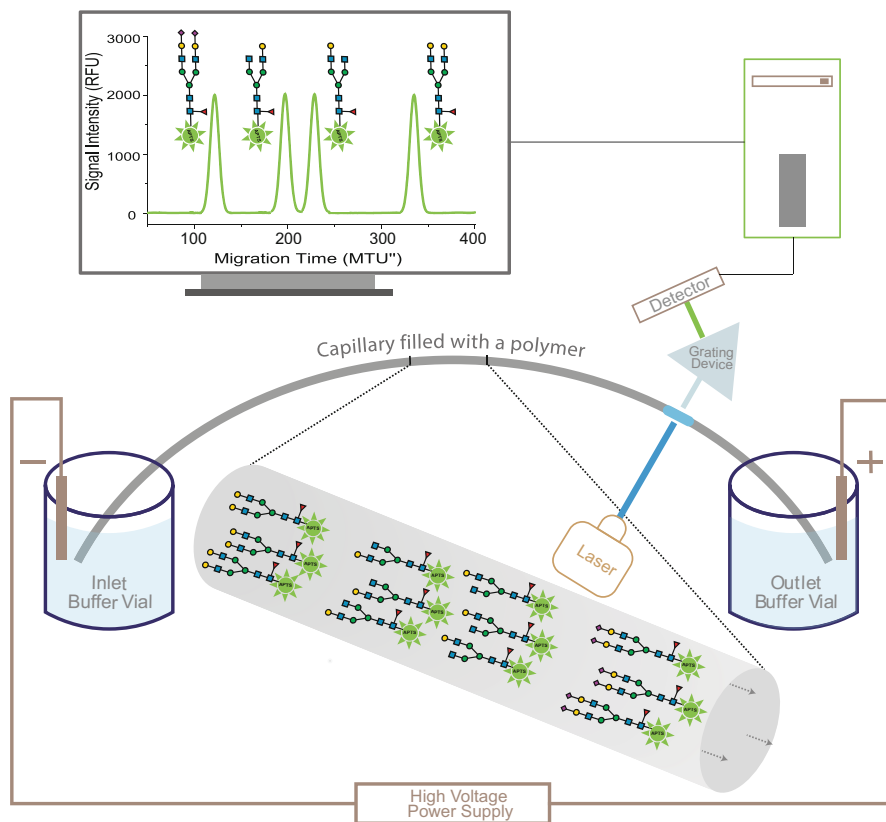


Fig. 4.1 Common separation of glycans utilizing an xCGE-LIF-based DNA sequencing system. High injection voltage is applied for a brief period, causing sample (glycans labeled with a negatively charged fluorescent dye, e.g., APTS) to enter the inlet of the gel-filled capillary by electromigration (electrokinetic injection). The electric field is applied under reverse polarity (cathode at injection and anode on the detection side) so that anions (i.e., negatively charged labeled glycans) migrate towards the detection window. As demonstrated, fluorescently labeled glycans are separated, based not only on the differences in their mass-to-charge ratios but also on their hydrodynamic volumes. Although sequencing systems benefit from multi-capillary format, only one capillary is presented here for simplicity. The x-axis of the schematic electropherogram is given in double aligned migration time units (MTU''). The signal intensity of the y-axis is given in relative fluorescent units (RFU). Symbolic representation of *N*-glycan structures follows the guidelines of Symbol Nomenclature for Glycans (SNFG) (Varki et al. 2015)

4.3 Performance, Benefits, and Potentials of Capillary (Gel) Electrophoresis C(G)E

CE provides remarkable separation efficiencies and resolution in a very short time, which makes this technology appealing for the analysis of structurally complex and diverse molecules like glycans. Recent work has shown that CE can separate even

challenging positional and linkage isomers in one single analysis run. For example, the method is capable of distinguishing even carbohydrate position in a glycan structure [e.g., nonreducing terminal residue on α 1–3 arm from terminal residue on α 1–6 arm of the core structure (see Fig. 4.2b, c) (Hennig et al. 2016; Schwedler et al. 2014a, b; Chen et al. 2017; Huang et al. 2017; Guttman et al. 2015)], along with position and linkage of fucose (*Fuc*) [e.g., α 1–6 core Fuc from α 1–3/1–4 Fuc on antenna *N*-acetylglucosamine (*GlcNAc*) or α 1–2 Fuc on galactose (Hennig et al. 2016; Konze et al. 2017; Thiesler et al. 2016; Weiz et al. 2016; Schwedler et al. 2014a)], type and linkage of sialic acids (*Sia*) [e.g., *N*-acetylneuraminic acid (*Neu5Ac*) from *N*-glycolylneuraminic acid (*Neu5Gc*), see Fig. 4.3d (Abeln et al. 2017), and α 2–3 from α 2–6 (Hennig et al. 2016; Konze et al. 2017; Thiesler et al. 2016; Donczo et al. 2017; Meininger et al. 2016)], and linkage of galactose (*Gal*) [e.g., β 1–3 Gal from β 1–4 Gal (Konze et al. 2017; Thiesler et al. 2016; Schwedler et al. 2014a; Muñoz et al. 2019)]. This feature becomes especially advantageous when thinking about the importance of determining immunogenic α -Gal and Neu5Gc on glycoprotein therapeutics (Chung et al. 2008; Teranishi et al. 2002; Van der Linden et al. 2000). Additionally, the fact that only α 2–6, and not α 2–3 sialic acid, affects the anti-inflammatory activity of IgG antibody (Anthony et al. 2008) makes it crucial to have a method capable of their differentiation.

Besides speed, resolution, sensitivity, and simplicity, compatibility with a wide range of samples is one of the biggest advantages of C(G)E. Due to the robustness of the method, only a modest sample purification is necessary. Large amounts of cell debris (Konze et al. 2017; Thiesler et al. 2016; Abeln et al. 2017) or other impurities like large excess of fluorescent labeling dye (Croset et al. 2012) will not prevent a successful analysis. For comparison, in LC-based analytics, such cell debris can block the column or at least accumulate inside of it, resulting in a significant decrease of separation power. Consequently, a costly column exchange or a time-consuming column cleaning procedure is needed. In contrast, in CGE, the separation matrix (polymeric gel) is exchanged after each run; thus impure samples are not problematic in terms of clogging or sample carryover. Even sample clean-up after exoglycosidase digest—for LC an obligatory step—can be omitted, further reducing analysis time and workload (Szigeti and Guttman 2017).

Moreover, CE analysis is beneficial when enormous differences in analyte concentrations are present in one sample. For example, it could be shown by Kottler et al. that lactose, which represents up to 85% of the free oligosaccharides in human milk, did not interfere with the analysis of other more complex but low abundant human milk oligosaccharides (*HMOS*) (Coppa et al. 1993). While in xCGE-LIF the lactose peak appears without any peak shape deformation, even when injected in amounts far above the linear detection range, for LC-based methods, lactose needs to be depleted from the milk samples before HMOS analysis to avoid massive peak broadening and tailing as a result of column overload (Kottler et al. 2013). Similar problems might be faced when using mass spectrometry (*MS*).

After reviewing all the given advantages, an important aspect of the *N*-glycan analysis is the costs. Resources in academia and industry are often quite limited, especially if hundreds or thousands of samples need to be analyzed in a short time—

i.e., in real high-throughput. Here glycan analysis by xCGE-LIF, utilizing DNA analyzers originally built for Sanger sequencing, outperforms conventional LC and MS approaches. With low material costs (due to minimal sample preparation) and low operating costs (low maintenance, no organic solvents and solvent disposal costs), the overall cost are significantly lower for xCGE-LIF (Huffman et al. 2014; Mahan et al. 2015) than for LC or LC–MS. Moreover, data analysis and interpretation became user-friendly with available software solutions (see section “Data Analysis and Interpretation”), further reducing expenses for hands-on time and expertise.

Finally, C(G)E-based methods were also successfully applied for the absolute quantification of carbohydrates (Sarkozy et al. 2021; Eussen et al. 2021), which completes the wide field of applications. Taken together, unprecedented separation together with low costs, robustness, speed of analysis, multiplexing capability, high sensitivity, and nano- to femtoliter injection volumes make CE a veritable competitor to other more traditionally used techniques for glycan analysis.

4.4 Data Analysis and Interpretation

One way to obtain structural information and assign glycans to peaks is through co-injection of glycan standards, which are commercially available and fully characterized (Guttman et al. 1996a, b; Guttman and Herrick 1996; Reusch et al. 2014). In a so-called spiking experiment, the glycan is indirectly identified by the height increase of an often perfectly Gaussian-shaped CE peak. Unfortunately, only a very limited number of glycan standards are available on the market. A more general approach for the identification of glycans in a separation-based method with spectroscopic detection relies on comparing migration times (for CE separations) between a sample and a glycan database (*DB*). In this case, a fully characterized glycan standard (pure or inside of a complex mixture) is analyzed once, and its migration time is stored inside a glycan *DB*. The *DB* enables identification for all further analyses without the need for spiking the glycan standard again. Accordingly, only glycan structures with known migration times can be identified. However, the buildup of a glycan *DB* requires a very reliable CE setup and method with very stable and reproducible migration times.

A long-term stable migration time, independent from instrument, operator, and lab, in combination with a *DB*, comprising a broad variety of glycan structures, eases the structural annotation. Several research groups tackled this problem in a similar way. By running an accompanying oligosaccharide standard (e.g., a glucose ladder or a set of single oligosaccharides) for each sample or set of samples, the migration time can be aligned to this standard, resulting in a standardized time axis. Hence, the migration time of individual glycan peaks can be given in standardized migration time units (*MTU*). The *MTU* of a peak can now be used to search inside a dedicated *DB* to assign the corresponding glycan structure. Often a glucose ladder is used as an external alignment standard (in a separate run), resulting in a standardized migration

time axis in Glucose Units (*GU*) (Guttman et al. 1996a; Guttman and Herrick 1996; Mittermayr et al. 2013; Laroy et al. 2006; Mittermayr and Guttman 2012; Liu et al. 2007). Recently, it was shown that a co-injection of a bracketing triple internal standard (maltose, maltotriose, and maltopentadecaose; by Agilent) could negate the need for additional glucose ladder run (Jarvas et al. 2016)—at least for simple samples and regarding short term repeatability. As bigger variations of migration times in-between the bracketing standard can occur (region of interest), Hennig et al. use a patented orthogonal double alignment, combining the standard bracketing approach and the ladder approach (Hennig et al. 2015, 2016; Huffman et al. 2014), resulting in correspondingly double aligned migration time units (*MTU''*). An alignment to the bracketing standard, labeled with the same fluorescent dye as the sample, is complemented with an orthogonal alignment to a DNA base pair standard, labeled with a different dye. Both standards are co-injected with the sample and detected in separate spectral traces. The additional DNA ladder-supporting points for the alignment significantly improved the (long-term) stability of the migration times, which allowed to build up a large *N*-glycan DB with more than 400 structures (glyXbase™ by glyXera). Recently, the glycan analysis software glyXtoolCE™ (by glyXera) was developed (Hennig et al. 2011b, 2016; glyXera 2021), which automates migration time alignment together with an instant structural assignment and furthermore provides the background adjustment raw data smoothing, peak picking, integration, relative quantification and sample comparison (Hennig et al. 2011b, 2016; Behne et al. 2013). Similar logic is behind the recent approach from Feng et al. (2017); however, alignment was performed in a non-automated fashion and with only a single normalization point to an internal glycan standard. Related bioinformatics tools such as GUCal were developed that can carry out GU value calculation in an automated fashion and concomitantly search through the database (at the moment comprised of 92 structures, available at www.glycostore.org) (GU database) for structural assignment (Jarvas et al. 2015, 2018). All these developments facilitate data processing and interpretation and make CE an easy-to-use high-throughput tool.

Nevertheless, while spiking experiments and database comparison give a strong indication of the glycan structures, they do not fully confirm them. Also, alternative approaches, when applied alone, do not allow full structural elucidation of glycans. For example, with single-stage MS, glycan composition can be estimated based upon the addition of monosaccharide constituent masses. However, glycans with different structures but identical monosaccharide composition result in identical mass values and cannot be distinguished with MS. Even with tandem mass spectrometry (*MS/MS*), isobaric stereoisomers [like hexoses, galactose, and mannose (*Man*)], positional isoforms, and the different types of glycosidic linkages are difficult or impossible to determine. Here chromatographic and electrophoretic separations are advantageous with their ability to resolve closely related positional and linkage glycan isomers. Nevertheless, the structural variety of glycans is enormous, and resolution capabilities are not indefinite. Thus, what appears as a single peak often comprises a mixture of glycans (multi-structure peak). For that reason, also glycan analytical methods based on chromatographic and electrophoretic

separation need a second dimension to provide correct and complete sample structural information. Thus, they are often complemented with an additional technique, such as MS or exoglycosidase sequencing. Principles, benefits, applications, and some limitations of exoglycosidase sequencing are outlined in the section below. A description of different CE and MS coupling possibilities, together with the overview of recent CE–MS-based glycomics studies, is provided in section “Coupling Capillary Electrophoresis with Mass Spectrometry.”

4.5 Exoglycosidase Sequencing of Glycans

Exoglycosidases are enzymes that cleave the terminal carbohydrate monomers on the non-reducing end of a glycan. They can be highly selective for specific monosaccharide types (e.g., Gal, Fuc, or Man), linkage orientation (α or β) or the position of the glycosidic linkage (e.g., 1–3, 1–4, or 2–3). Apparently, the specific enzymatic cleavage of monosaccharide residues serves as the most efficient approach for glycan structural elucidation in CE- and LC-based analysis because no additional equipment is needed. The conversion of a glycan by an exoglycosidase is harnessed with CE by analyzing the sample before and after the treatment. Digest-induced charge, size, and shape changes can be observed in CE as a change in migration time. Depending on enzyme specificity, information on monosaccharide type and the number of cleaved residues, sequence, or even linkage and anomericity can be obtained, as detailed shown by Cajic et al. (Thiesler et al. 2016) (in the supplemental material). Analysis of peak positions and relative peak areas before and after an exoglycosidase digest is therefore a means of exhaustive structural annotation. These digests can be conducted following four different strategies. First option is sequencing in a parallel fashion (Hennig et al. 2016; Thiesler et al. 2016; Feng et al. 2017), with the sample being evenly split into one aliquot per enzyme and simultaneous analysis of enzyme-treated aliquots. Second option is sequencing with parallel exoglycosidase treatments, mediated by (different) carefully designed mixtures of exoglycosidases (Szigeti and Guttman 2017; Guttman and Ulfelder 1997; Guttman 1997). Through multiple combinations of exoglycosidases (parallel enzyme array), glycans are sequenced down to their Man3-core structure (Prime and Merry 1998; Rudd and Dwek 1997). Instead of splitting the sample, it is possible to apply individual enzymes (third option) or mixtures of enzymes (fourth option) sequentially to the same sample, followed by an analysis of each step (Szigeti and Guttman 2017; Guttman et al. 2015; Mechref et al. 2005; Ma and Nashabeh 1999). This sequential treatment of a single sample is often performed when the sample amount is limited or when the sample complexity is too high. It is common practice, e.g., to reduce sample complexity by sialidase treatment before peaks are annotated (Muñoz et al. 2019; Vanderschaeghe et al. 2010; Zhuang et al. 2007, 2011). Each strategy has its advantages and drawbacks, parts of which have been already addressed elsewhere (Mittermayr et al. 2013; Prime and Merry 1998; Holland et al. 2017) and are consequently not discussed here.

Approaches to enzymatically elucidate a glycan structure in a glycan mixture are quite diverse. For instance, it was shown that the reaction of a sequential enzyme treatment could be performed inside the CE autosampler, with the sample being injected from the enzyme reaction vial directly into CE (Szigeti and Guttman 2017). Another interesting alternative to offline methods is the incorporation of enzymes into the capillary (online digest). In-capillary enzymatic digests are a rapid option, with incubation times down to only a few minutes. By passing an enzyme plug inside the capillary, the digest of the samples is accomplished during the separation process itself (mixing via polarity switching, stopped flow or low flow incubations) (Luo et al. 2010; Holland et al. 2017; Archer-Hartmann et al. 2011a, b; Yagi et al. 2011; Gattu et al. 2017; Yamagami et al. 2017). The thermally tunable phospholipid nanogels are especially attractive due to the reported enhancement of the stability and performance of exoglycosidase enzymes (Holland et al. 2017; Yamagami et al. 2017). However, in-capillary endeavors are sometimes incompatible with certain enzymes (Yagi et al. 2011; Yamagami et al. 2017) and often accompanied by a loss in separation efficiency (Archer-Hartmann et al. 2011a, b).

Although exoglycosidases can be combined with many other methods, due to already praised advantageous features of CE, combining the specificity of exoglycosidase enzymes with the strengths of CE seems most appropriate to determine glycan structures unambiguously and with minimum effort. Because of the high resolving power of CE, multi-structure sequencing of a complex glycan pool can be performed requiring no prior isolation of the individual glycans, resulting in significant labor and time savings. Even if enzymes are cleaving terminal residues without (much) specificity regarding linkage or position, CE can still provide this information due to its high separation efficiency. Thanks to the very good reproducibility of the peak areas, even small differences in glycan abundance can be detected reliably so that a digest-induced shift of a minor structure under a peak (less than 5% of total peak area) can be successfully tracked. Because of the electrokinetic loading system and highly sensitive LIF detection, only a very low amount of sample is required per injection, which means that most of the sample remains for other analyses. High-throughput capabilities obtained from multi-capillary CE formats and fast separations qualify CE as an ideal screening method of, for example, optimal reaction conditions or batch-to-batch variations. Excellent migration time stability of some CE methods allows migration time-database matching for original and exoglycosidase digested products, making analysis much faster and more efficient. All these characteristics make CE in conjunction with exoglycosidase sequencing a valuable tool for detailed glycan analysis.

Despite all acknowledged benefits, the use of enzymes for determining the glycan structure requires great care and expertise and should involve a large dose of healthy skepticism about any deduced structure (Jacob and Scudder 1994). Even if certain sugar residues are exposed at the reducing end of the glycan, these residues are not always removed by the exoglycosidases with the corresponding specificities. This resistance can be due to the strict linkage specificities of the exoglycosidases, due to steric hindrance of neighboring sugar residues or due to the attached (fluorescent) label. For example, hydrolysis of bisecting GlcNAc in human IgG *N*-glycans upon

hexosaminidase treatment or the core Fuc upon bovine kidney α -fucosidase treatment might be incomplete or completely impeded (Mittermayr et al. 2013; Laroy et al. 2006; Guttman and Ulfelder 1997; Jacob and Scudder 1994; Kamerling and Gerwig 2007; O'Flaherty et al. 2017). In addition, used enzymes may have additional selectivity for other structural features, such as local and nonlocal branching. For example, β -galactosidase can hydrolyze β 1–3,4,6-linked Gal exposed at the non-reducing end of an antenna without Fuc attached to the subterminal GlcNAc. If Fuc is attached to the antenna GlcNAc, the β -galactosidase will not hydrolyze the Gal (Yu et al. 2011). Hence, a particular exoglycosidase will cleave a terminal monosaccharide only if all its specificity requirements are met. However, the purity and composition of both glycan material and used reagents can greatly affect enzyme activity and pose an additional difficulty for accurate and reliable structural assignment (Jacob and Scudder 1994). For example, variation in enzyme activity has been observed between different batches from one vendor and different sources (vendors) of the same enzyme. Enzyme activities and side activities are often tested only on artificial p-nitrophenyl glycosides or other simple substrates, even though activity on natural complex glycans can significantly differ (Kobata 2013). Additionally, vendor-added salts or other additives have the potential to interfere with different aspects of the analysis. For instance, electrokinetic injection in CE is sensitive to high salt content originating from the non-volatile digestion media (Mittermayr et al. 2013; Laroy et al. 2006), which is usually unavoidable with commercial enzymes. Finally, a major issue can be the purity of the exoglycosidase. Many of the enzyme preparations contain a certain amount of contaminant enzymes, ranging from very low to unacceptably high (Jacob and Scudder 1994; Kamerling and Gerwig 2007). Therefore, even when a positive or negative result is obtained by digestion with exoglycosidases, it does not necessarily confirm the presence or absence of the corresponding sugar residues at the non-reducing end of glycan, respectively (Kobata and Takasaki 1992). Thus, using dedicated positive and negative controls for each enzyme reaction is unavoidable.

Despite the abovementioned considerations, it was shown that, when used cautiously, exoglycosidase can effectively elucidate even subtle changes in glycan structures, including linkage type (e.g., α 2–3 versus α 2–6 Sia or β 1–3 versus β 1–4 Gal) (Callewaert et al. 2001; Hennig et al. 2016; Konze et al. 2017; Thiesler et al. 2016), anomericity (e.g., immunogenic α -Gal versus β -Gal) (Abeln et al. 2017; Szabo et al. 2012; Yagi et al. 2012) and position on glycan (e.g., core Fuc versus Fuc on antenna GlcNAc or Gal) (Konze et al. 2017; Thiesler et al. 2016; Meininger et al. 2016; Liu et al. 2007; Zhao et al. 2014)—a complexity which is often inaccessible by methods other than nuclear magnetic resonance (NMR) spectroscopy.

4.6 Coupling Capillary Electrophoresis with Mass Spectrometry

Combining CE, one of the most effective isomer separation tools, with the information-rich MS technique is a mutually advantageous and powerful alliance for in-depth glycan analysis. Two setups are widely applied: the direct linking of CE to MS, the so-called online CE–MS, or a time-separated analysis, the offline CE–MS, in serial or parallel mode. For a serial offline approach, a CE instrument can be modified to spot the eluent from the capillary directly onto a target-plate for matrix-assisted laser desorption ionization with time-of-flight MS (*MALDI-TOF-MS*) detection (Suzuki et al. 1997). Consequently, separation is done by CE and identification by MS. Furthermore, parallel offline approaches are gaining popularity, especially for MS-based glycopeptide or intact glycoprotein analysis (glycoproteomics). Here, the vast diversity of protein/peptide–glycan combinations (driven by the complexity of glycans) is dramatically increasing the computing time for the interpretation of MS spectra (Thaysen-Andersen and Packer 2014; Yang et al. 2017). For reduction of computing time, an initial global characterization of the *N*-glycome can be performed by CE. The resulting glycan list is used to search against a targeted set of defined *N*-glycan structures as variable protein/peptide modifications (Thaysen-Andersen and Packer 2014; Parker et al. 2013; Lebede et al. 2021; Pralow et al. 2021; Pioch et al. 2018). This pre-knowledge of attached glycan structures greatly reduces the search space, which in turn significantly decreases the computing time. This allows relatively fast and in-depth analysis of quite complex samples, even with demanding glycoproteomic approaches.

The major advantage of online CE–MS is that glycans are identified by both their differential migration times and their molecular masses and/or fragmentation patterns in one analysis. The most common interface used for the transfer of glycans from the liquid phase of CE to the gas phase of MS is electrospray ionization (*ESI*) since this soft ionization method allows the direct transfer of the glycans from separation capillary to MS and is rather easy to implement. A detailed description of the advantages and drawbacks of the different interfacing options is beyond the scope of this chapter and has been already provided elsewhere (Simó and Cifuentes 2005; Maxwell and Chen 2008; Zhong et al. 2014; Zhang et al. 2017), together with the comprehensive overview of CE–MS-based glycomics studies (Pioch et al. 2012; Nakano et al. 2011; Mechref and Novotny 2009; Mechref 2013; Lindenburg et al. 2015; Dotz et al. 2015).

Although the CE–MS technology was introduced already 30 years ago (Olivares et al. 1987; Smith et al. 1988a, b) and continues to advance ever since, there has been relatively little work performed on merging CE with online electrospray MS for analysis of released glycans, in particular, when compared to the hyphenated LC–MS techniques. Coupling the high resolving power of CE and structural information of MS in one system often comes at the expense of resolution, sensitivity, analysis time, reproducibility and/or robustness. Very often, the separation conditions giving the unmatched separation efficiency to CE are detrimental to MS performance and

vice versa. For example, gels and buffers often used in CE because of resolution improvement are not volatile and are not suitable for CE–ESI–MS since they often suppress the ionization of the analyte, yielding poor MS sensitivity or even clog the system. On the other hand, the choice of volatile, “MS-friendly” buffers can not only affect reproducibility and analysis time but even negatively impact separations. This necessary compromise between optimal MS performance and elevated CE separation efficiency, plus its lacking robustness, are the major reasons that CE–MS has still not been widely adopted as a routine method for glycan analysis. Another concern to be addressed is that with all the limitations (small sample loads and consequent low sample concentration, sample dilution by the sheath-liquid introduction, labeling incompatibility issues, and ionization efficiency considerations) inherent in CE–MS analysis, obtaining detailed linkage and/or positional information by MS/MS is often challenging.

Even though we cannot yet talk in terms of widespread acceptance, still some efforts to make CE–MS a more viable approach in the field are ongoing. Applications are mainly limited to the use of capillary zone electrophoresis (CZE) coupled to MS for protein characterization and glycan identification. Besides the analysis of intact glycoproteins, CZE–MS is used to characterize side-specific microheterogeneity on glycopeptide level (Pioch et al. 2012; Lindenburg et al. 2015; Dotz et al. 2015). The analysis of native or derivatized glycans is often performed on complex samples (e.g., plasma *N*-glycomes), taking advantage of the good separation performance of CE (Snyder et al. 2017; Lageveen-Kammeijer et al. 2019; Huhn et al. 2012; Jayo et al. 2012). CZE–MS-based characterization of single protein glycosylation is performed less frequently (Jayo et al. 2014) and focuses mainly on monoclonal antibodies (*mAbs*) (Pioch et al. 2012; Mechref 2013; Dotz et al. 2015; Gennaro and Salas-Solano 2008; Bunz et al. 2013a, b).

4.7 Latest Developments: Miniaturization of CE Systems— Microchip CE

Easy-to-use technologies that allow rapid and efficient screening in a compact format at the best cost are in great demand, especially in the biopharmaceutical industry. Miniaturization of the electrophoretic process onto microchips (microchip CE) holds a great promise to meet all these needs. No other technology seems better suited for miniaturization owing to two main hallmarks of CE. Firstly, sophisticated pumping systems are not necessary since the separation is driven by an applied electric field. Secondly, separation efficiency depends primarily on the strength of the electric field applied along the separation capillary and not on its length (Holland et al. 1997). Most of the commercial microchip-based systems employ relatively short separation channels (<10 cm, down to few millimeters) and modest electric field strengths (<500 V/cm). However, in practice, microchip CE systems show lower separation efficiencies compared with standard CE capillary separations,

primarily due to their shorter channel lengths. Even with this apparent disadvantage, these devices provide sufficient resolution to separate and compare all major *N*-glycans found on IgG and mAbs in 60 s or less (Vanderschaeghe et al. 2010, 2013; Smejkal et al. 2010; Primack et al. 2011; LabChip Microfluidics 2018), illustrated in Fig. 4.2a.

The misconception that complex *N*-glycan samples are not amenable to separation by microchip CE has been proven wrong by recent research work. To increase the component resolution for these microfluidic separations, longer separation channels need to be fabricated on microchips. To keep the overall dimension of a chip turns had to be integrated into the channels without introducing significant sample dispersion. With these longer separation channels (>20 cm) and higher electric field strengths (>1000 V/cm), microfluidic devices are now able to rapidly and efficiently separate *N*-glycans derived even from complex samples in less than 3 min (Zhuang et al. 2007, 2011; Snyder et al. 2016, 2017; Mitra et al. 2012, 2013, 2016). Although they have great resolving power, there is often higher variability in migration times when higher separation field strengths are applied (Mitra et al. 2012).

Figure 4.2 shows a direct comparison of separations of bovine IgG-derived *N*-glycans performed by microchip CE, xCGE-LIF equipped with 50 and 22 cm capillary-arrays, and hydrophilic interaction high-performance liquid chromatography (*HILIC-HPLC*). Miniaturization of the entire electrophoretic process resulted in a reasonably satisfactory separation of the major *N*-glycan peaks in seconds, as opposed to a much better separation for xCGE-LIF and *HILIC-HPLC*, but in minutes. However, for glycoproteins with a low glycan complexity, the capillary length in conventional CE and CGE systems can be further shortened without considerable loss of separation power, as shown for bovine IgG analyzed by xCGE-LIF with 50 and 22 cm capillaries (Fig. 4.2b, c). An xCGE-LIF instrument equipped with a 16-channel array with capillaries of 22 cm in length has an effective separation time of 32 s per sample (8.5 min/16 samples), which is close to 22.5 s obtained by the chip-CE instrument, but with a significantly better separation of the *N*-glycan peaks. Keeping the multiplexing aspect of xCGE-LIF in mind, with its possibility to run up to 96 samples in parallel, it becomes obvious that there is potential to further increase the throughput of microchip CE.

Thus, despite the mentioned advancements, “Lab-on-a-chip” technology—incorporating both sample preparation and analysis onto the same microfluidic devices with a minimum hands-on time and being sufficiently simple for non-experts—still remains an ultimate goal. But first, the current generation of miniaturized systems has yet to demonstrate benefits in cost and performance, compelling enough to make them seriously competitive with conventional benchtop-scale CE technology. This is reflected in the current market volatility. The reliable availability of equipment, consumables, or support can often not be guaranteed. Therefore, low-cost, reliable and highly efficient microchip CE systems are expected to encourage greater use of this technology in the glycomics field.

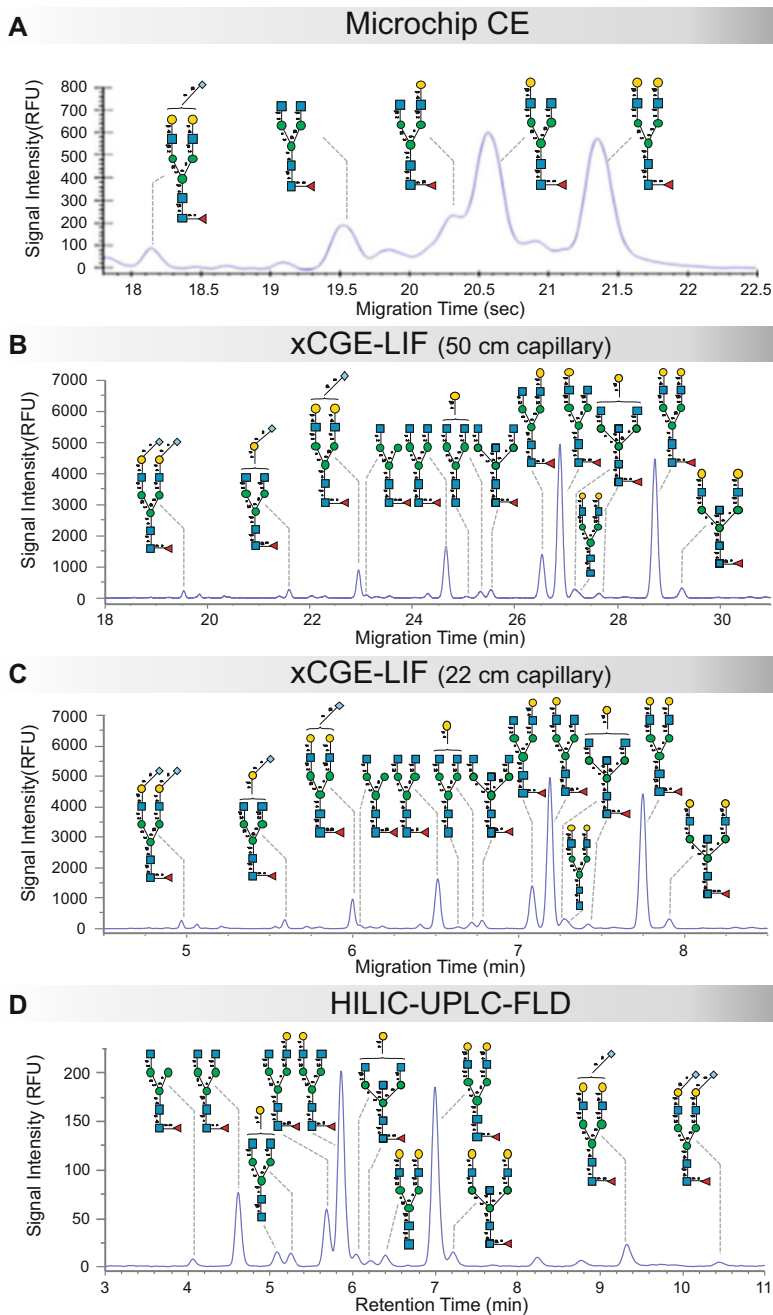


Fig. 4.2 Bovine IgG *N*-glycome analysis on the (a) PerkinElmer LabChip GXII Touch (microchip CE), (b and c) glyXera glyXboxCE™ built on Applied Biosystems Genetic Analyzer 3130 (xCGE-

4.8 Application of C(G)E for Immunoglobulin Analysis

Compared to common LC- and MS-based approaches, CE-based glycan analysis approaches are lagging far behind with respect to the number of applications and publications. This is largely due to the existing obstacles that needed to be tackled, including missing kits for sample preparation and non-flexible, difficult to handle or often unstable instruments. However, as discussed in detail in the previous sections, all these initial difficulties are now solved. Since stable and easy-to-handle DNA analyzers were adopted for *N*-glycan analyses by Callewaert and coworkers (Callewaert et al. 2001; Laroy et al. 2006), this approach gained popularity in the field of glycomics. Moreover, the availability of commercial analysis kits and instrumental solutions [e.g., by glyXera, PerkinElmer, ProZyme/Agilent, Sciex and Thermo Fisher Scientific (glyXera 2020; Thermo Fisher Scientific 2018; ProZyme 2018; SCIEX 2018; PerkinElmer 2018)], including software and glycan databases, made C(G)E-based methods appealing to the scientific community, as well as to the biopharmaceutical industry. This broader acceptance can be additionally attributed to the separation power of C(G)E and the ability to resolve complex glycan mixtures and to separate positional and linkage isomers. The following paragraphs are intended to highlight significant applications of C(G)E to the analyses of *N*-glycans in the science and biopharmaceutical industry and some new, exciting possibilities with a special emphasis on immunoglobulins.

IgG The C(G)E-based analysis of IgG *N*-glycosylation is well established (Reusch et al. 2014, 2015a; Routier et al. 1998) and was already applied to big cohorts (Huffman et al. 2014). Small sample quantities required for the xCGE-LIF-based analysis turned out to be quite beneficial for studies with limited sample availability, e.g., for the analysis of mouse IgG *N*-glycosylation (Patenaude et al. 2020; Schaffert et al. 2020). Consequently, this method can be applied to all kinds of mammalian samples, from large to small. As an example, Fig. 4.3 shows a comparison of the IgG *N*-glycans derived from various mammalian species, namely human, cow, mouse, dog, and rabbit. Human and bovine (cow) IgG-derived *N*-glycans show quite some similarities, despite the absence of the Neu5Gc sialic acid type in humans, respectively of Neu5Ac in cow (Raju et al. 2000). However, more dominant are the differences between the species. Their IgG *N*-glycomes vary in composition and



Fig. 4.2 (continued) LIF), (d) Dionex Ultimate 3000RS UPLC system equipped with Waters ACQUITY UPLC Glycan BEH Amide column. For (a), labeled bovine IgG *N*-glycans were prepared using the PerkinElmer Glycan Profiling Assay Reagent Kit (PerkinElmer 2018). For (b) and (c), APTS-labeled *N*-glycans were prepared from bovine IgG following a published procedure (Hennig et al. 2016; Huffman et al. 2014). For (d), *N*-glycans were enzymatically released from bovine IgG, AB-labeled (Ruhaak et al. 2010c) and subsequently purified (Ruhaak et al. 2008, 2012) before measurement by HILIC-UPLC-FLD. The signal intensity of the y-axis is given in relative fluorescence units (RFU). Symbolic representation of *N*-glycan structures follows the guidelines of Symbol Nomenclature for Glycans (SNFG) (Varki et al. 2015)

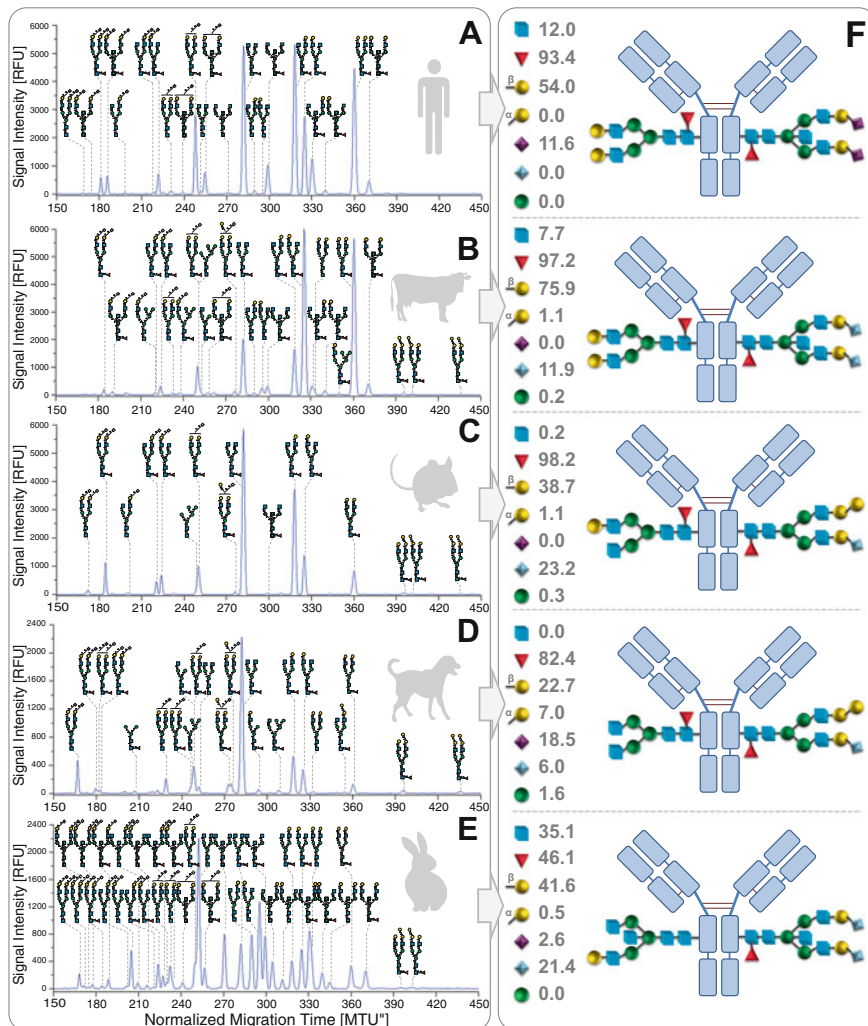


Fig. 4.3 Species-specific IgG *N*-glycosylation analyzed by xCGE-LIF. xCGE-LIF generated fingerprints of APTS-labeled *N*-glycans derived from human IgG (a), bovine IgG (b), mouse IgG (c), dog IgG (d) and rabbit IgG (e) (Vendors: Dog IgG—Abcam, other IgGs—Sigma-Aldrich). APTS-labeled *N*-glycans were prepared using the glyXprep⁴⁸ kit (glyXera 2020) by carefully following the kit instructions. Data processing was performed using glyXtoolCETM (glyXera 2021). Data processing comprised alignment of migration times to two orthogonal internal standards, resulting in a double aligned x-axis in migration time units (MTU^{''}). The signal intensity of the y-axis is given in relative fluorescence units (RFU). *N*-glycan structures were assigned via database matching using glyXtoolCETM (in combination with glyXbaseTM) and confirmed by exoglycosidase sequencing as published by Thiesler et al. (2016). (f) Overview of IgG *N*-glycome characteristics with: blue square for bisected *N*-glycans, red triangle for core fucosylated *N*-glycans, yellow circle for glycans with terminal galactose in β 1–4 or α 1–3 linkage, purple diamond for glycans with terminal *N*-acetylneuraminic acid (Neu5Ac), light blue diamond for glycans with terminal *N*-glycolylneuraminic acid (Neu5Gc) and green circle for oligo-mannosidic *N*-glycans.

abundance of different glycans; from quite simple mixtures of glycans, with only a few structures (like the murine/mouse IgG in Fig. 4.3c), to extremely complex mixtures, with a wide range of structures, as shown in Fig. 4.3e for leporid (rabbit) IgG. Due to the big proportion of bisected *N*-glycans, in combination with a low degree of galactosylation and fucosylation, leporid IgG-derived *N*-glycans show one of the most complex glycosylation patterns (Taniguchi et al. 1985), which is still well resolved by xCGE-LIF, compared to results achieved by HILIC-UPLC analysis (Vainauskas et al. 2016). Additionally, the ability to separate Neu5Ac from Neu5Gc, as shown for canine (dog) IgG in Fig. 4.3d at about 180 MTU'', exposes the big potential of C(G)E-based glycan analysis techniques. As recombinant IgGs produced in different host cells is one of the major therapeutic agents to treat life-threatening diseases (Raju et al. 2000), fast and effective analysis of species-specific glycosylation differences by methods like C(G)E is becoming invaluable in this field.

Other Igs Humans have five distinct classes of Igs, namely IgA, IgD, IgE, IgG, and IgM. All Igs are comprised of two heavy and two light chains that are joined together by disulfide bonds. The glycosylation of Igs greatly varies between the different classes, ranging from only one conserved *N*-glycosylation site in IgG up to various *N*- and *O*-glycosylation sites for IgD (Maverakis et al. 2015; Clerc et al. 2016). It has long been known that aberrant IgA glycosylation causes IgA nephropathy and Henoch-Schoenlein purpura nephritis (Allen et al. 1995; Novak et al. 2007), and it was recently discovered that IgE sialylation is one regulator of allergic reactions (Shade et al. 2020). But, although Igs are the major component of the adaptive immune system, only little is known about the influence of their glycosylation, apart from the extensively studied IgG. This is partially due to current technological limitations and the complexity of their glycosylation (Maverakis et al. 2015).

Here, we show how to overcome these limitations by giving a comprehensive overview on *N*-glycosylation of all plasma immunoglobulins, analyzed by xCGE-LIF as shown in Fig. 4.4. In contrast to human IgG with incomplete sialylation (Fig. 4.4d), human IgA, IgD, IgE, and IgM show a high degree of sialylation—often in combination with a big variety of oligo-mannosidic *N*-glycan structures (Fig. 4.4a–c, e). In comparison to HILIC-HPLC results published in the early 2000s (Arnold et al. 2004, 2005; Mattu et al. 1998), the xCGE-LIF results display markedly better resolution, resolving structures appearing as multi-structure peaks in HILIC-HPLC. Guttman and coworkers achieved similar results using a CE-based



Fig. 4.3 (continued) Numbers indicate their approximated relative abundances in percent. IgG illustrations indicate the broad characteristics of the *N*-glycan structures attached to the species-specific IgGs. IgG illustrations are for visualization purposes only and do not reflect a real combination of *N*-glycans or their linkage position to the protein backbone. Symbolic representation of *N*-glycan structures follows the guidelines of Symbol Nomenclature for Glycans (SNFG) (Varki et al. 2015)

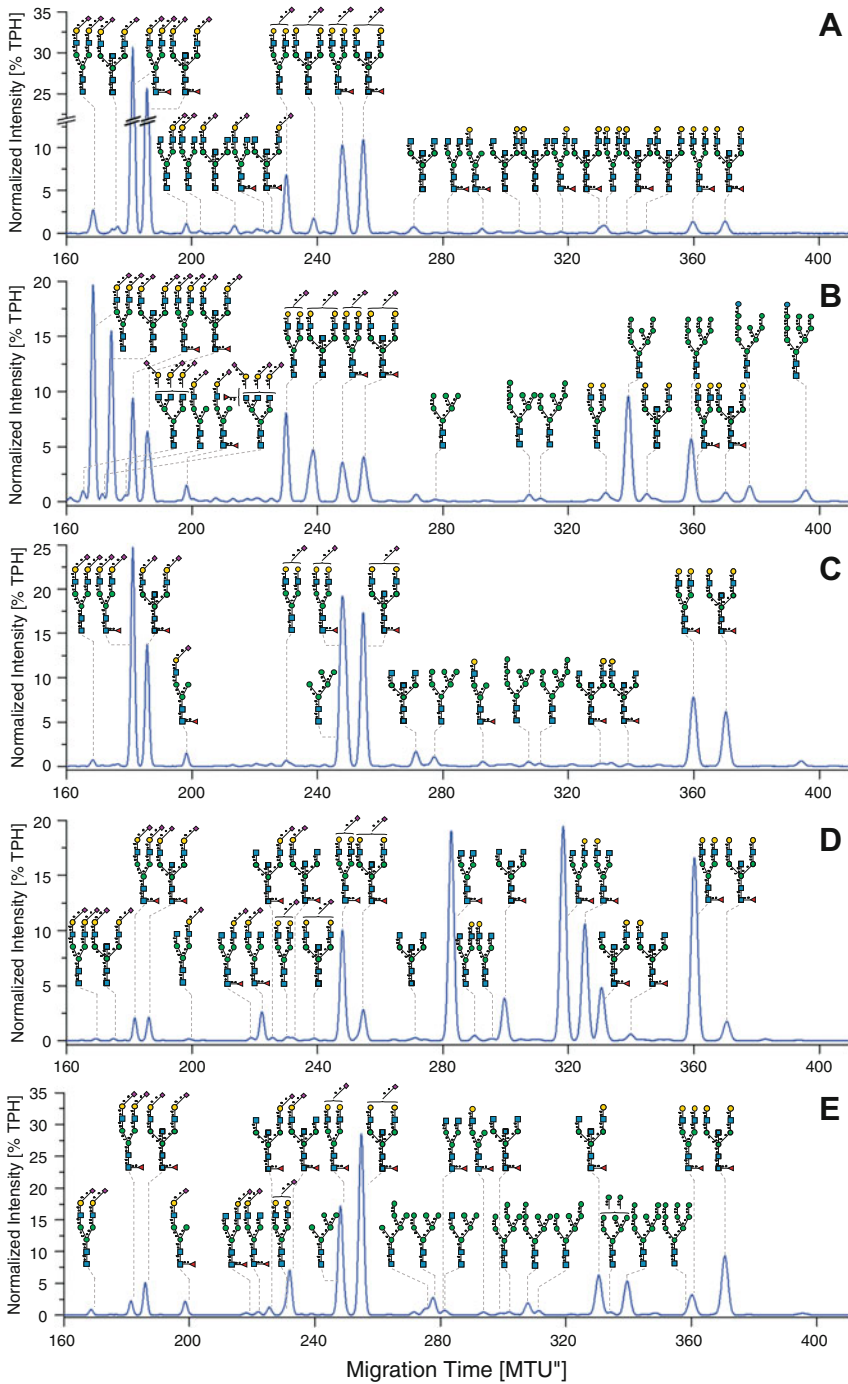


Fig. 4.4 *N*-glycan analysis of all human serum immunoglobulins by xCGE-LIF. xCGE-LIF generated fingerprints of APTS-labeled *N*-glycans derived from human IgA (a), IgD (b), IgE (c), IgG (d) and IgM (e) (Vendor: Abcam for IgA, IgD, IgE, and IgM; Sigma-Aldrich for IgG).

approach for the analysis of IgA from blood and saliva (Meszaros et al. 2020). They applied the method to biomedically relevant samples and found a link between altered IgA *N*-glycosylation and oral mucositis (Gebri et al. 2020). As the role of Ig glycosylation (besides IgG) is still not fully understood, xCGE-LIF might therefore simplify and speed up data generation and interpretation—thus, in the future promote interesting new findings when applied to big cohort studies.

Fab and Fc Antibody Domains A general approach in the field of glycobiology is to analyze released *N*-glycans of the entire IgG (the IgG *N*-glycome) (Huffman et al. 2014). However, *N*-glycans can appear at two different positions on IgG. The majority of *N*-glycans are attached to the conserved *N*-glycosylation site inside the constant (Fragment crystallizable, *Fc*) domain, while only a minor portion of *N*-glycans (up to 20%) might originate from the variable (Fragment antigen binding, *Fab*) domain of IgG (van de Bovenkamp 2019). The so-called Fab *N*-glycans differ from those attached to the Fc part, as the Fab portion possesses primarily highly sialylated and bisected complex type *N*-glycans (Clerc et al. 2016; van de Bovenkamp 2019), as shown in Fig. 4.5b (black curve), compared with the Fc-associated glycans with lower sialylation, as shown in Fig. 4.5b (blue curve). Having a global look at glycosylation of other human immunoglobulins (Fig. 4.4), the Fab glycosylation shows high similarity. The higher degree of sialylation in Fab-associated glycans of IgG might originate in part from selective removal of non-sialylated structures by the hepatic asialo-glycoprotein receptor (*HAGR*) (as for IgA, IgD, IgE, IgM, and all other blood proteins), resulting in a plasma glycoprotein typical glycosylation (Dalziel et al. 1999). Fc-associated glycans are less accessible (“hidden”), and accordingly not cleared from the bloodstream by *HAGR*. Furthermore, the presumably better accessibility of the IgG Fab glycosylation site to glycosyltransferases results in better processing compared to Fc glycans that are spatially localized inside the constant domain and not accessible to the same enzymes (van de Bovenkamp 2019).

Unfortunately, many big cohort studies of IgG glycosylation are focusing on released *N*-glycans and are not differentiating between IgG Fab- and Fc *N*-glycosylation (Huffman et al. 2014; Wang et al. 2017; Russell et al. 2017; Barrios et al. 2016; Trbojevic Akmacic et al. 2015; Lauc et al. 2013; Pučić et al. 2011), although recent studies suggest that the prevalence and complexity of Fab glycans might



Fig. 4.4 (continued) APTS-labeled *N*-glycans were prepared using the glyXprep⁴⁸ kit (glyXera 2020), by carefully following the kit instructions. Data processing was performed using glyXtoolCETM (glyXera 2021). Data processing comprised alignment of migration times to two orthogonal internal standards, resulting in a double aligned x-axis in migration time units (MTU^{''}). Peak heights were normalized to the sum of all peaks, resulting in a normalized intensity in % of total peak height (TPH). *N*-glycan structures were assigned via database matching using glyXtoolCETM (in combination with glyXbaseTM) and confirmed by exoglycosidase sequencing as published by Cajic et al. (Thiesler et al. 2016). Symbolic representation of *N*-glycan structures follows the guidelines of Symbol Nomenclature for Glycans (SNFG) (Varki et al. 2015)

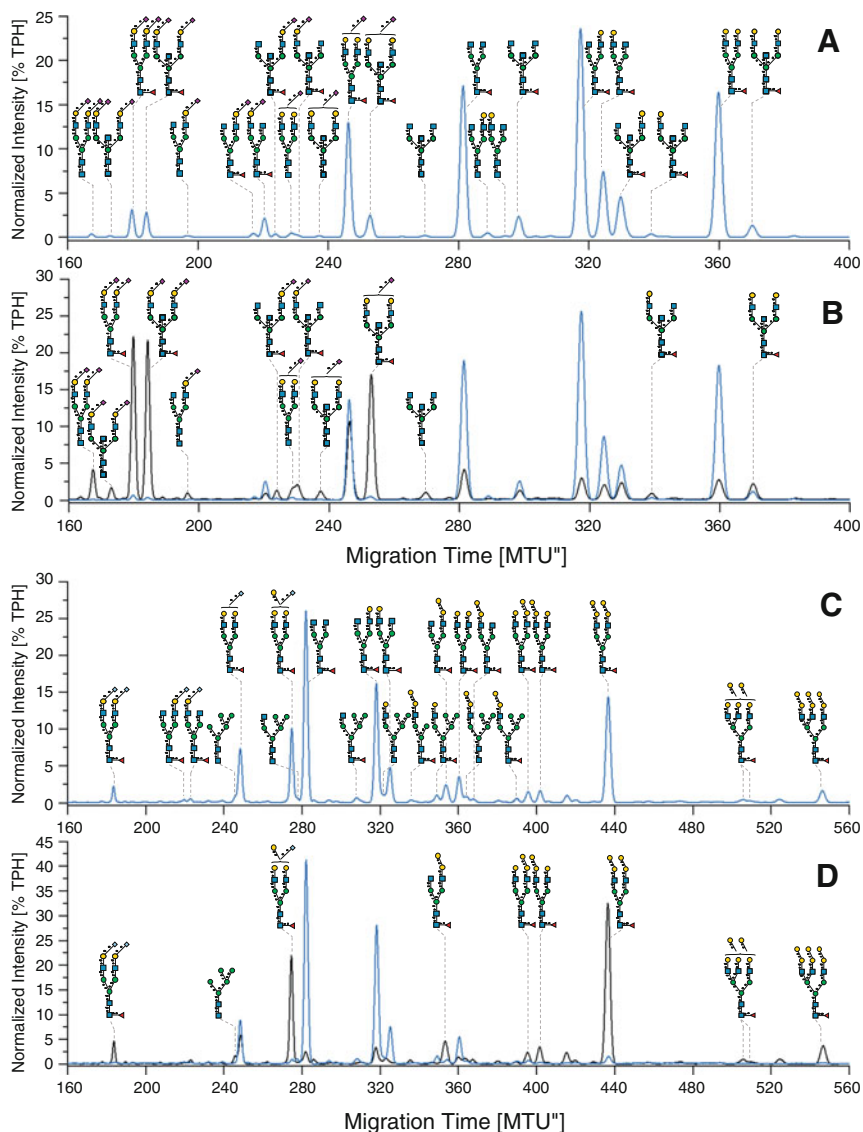


Fig. 4.5 Fab and Fc glycosylation analysis by xCGE-LIF, exemplarily shown for human IgG and the SP2/0 cell-derived mAb cetuximab (Erbix). xCGE-LIF generated fingerprints of APTS-labeled *N*-glycans derived from: whole human IgG (a), human IgG Fab (b, black curve), human IgG Fc (b, blue curve), whole mAb cetuximab (c), cetuximab Fab (d, black curve) and cetuximab Fc (d, blue curve) (human IgG was purified from normal control plasma, purchased at Affinity Biologicals; cetuximab was purchased at Evidentic). Fab and Fc part of human IgG and mAb cetuximab were purified and generated as published by Bondt et al. (2014). Release and APTS-labeling of *N*-glycans were performed, using the glyXprep^{4S} kit (glyXera 2020), by carefully following the kit instructions. Data processing was performed using glyXtoolCETM (glyXera 2021). Data processing comprised alignment of migration times to two orthogonal internal standards, resulting in a double aligned x-axis in migration time units (MTU''). Peak heights were

change for certain physiological and pathological conditions. On the one hand, sialylated Fab glycans are presumed to be protective during pregnancy (Bondt et al. 2014), while on the other hand, an increase in Fab glycans is associated with several autoimmune diseases, like rheumatoid arthritis and Sjögren's syndrome, or cancer like multiple myeloma (Kinoshita et al. 1991). These aspects show that a separate analysis of Fab and Fc glycosylation might be beneficial for a better understanding of immunological processes and that Fab and Fc glycans might be a promising biomarker for the early detection of various diseases. Here, especially C(G)E shows big potential because of its high sensitivity—enabling detection of even the lowest amounts of Fab glycans (Fig. 4.5b), and its speed (by multiplexing)—enabling higher sample throughput if needed.

Therapeutic Glycoproteins Since glycosylation of monoclonal antibodies (mAb) is a critical quality attribute (CQA), the detailed characterization and control of antibody glycosylation during the development process and later product life cycle is essential. Despite the ever-growing use of C(G)E-based methods for biopharmaceutical characterization, the number of publications in the field is still quite limited (Reusch et al. 2014; Croset et al. 2012; Bunz et al. 2013a; Bielser et al. 2020; Borza et al. 2018). Nevertheless, several studies showed that the CGE-based analysis results are quite comparable to the results obtained with conventional MS- and LC-based methods (Reusch et al. 2015a, b; De Leoz et al. 2020). As shown for the SP2/0 cell-derived mAb cetuximab in Fig. 4.5c, d, the overall results are quite comparable to the earlier analysis results achieved with HILIC-HPLC and MALDI-TOF MS (Qian et al. 2007), or ESI-TOF MS (Janin-Bussat et al. 2013). Here xCGE-LIF could also resolve the isomeric structures containing the immunogenic α -Gal from non-immunogenic β -Gal for Fc, Fab (Fig. 4.5d) and complete mAb *N*-glycosylation (Fig. 4.5c). After the discovery that the α 1–3 Gal epitope can cause an anaphylactic shock via an anti-oligosaccharide IgE-mediated reaction (Chung et al. 2008; Chinuki and Morita 2019), the absence of this epitope is intended for all newly developed therapeutic glycoproteins and biosimilars. Nevertheless, several previously commercialized mAbs are produced in murine hybridoma cell lines like NS0 and SP2/0, which do express the α 1–3 Gal epitope (Qian et al. 2007; Uçaktürk 2012; Stadlmann et al. 2008), as shown in Fig. 4.6a for the comparison of NS0-derived mAb ramucirumab (Cyramza) (blue line) and SP2/0-derived mAb cetuximab (Erbix) (black line). In contrast, the current dominant production cell lines are derived from Chinese Hamster Ovary (CHO) cells, which do not express the immunogenic α 1–3 Gal epitope, as shown in Fig. 4.6b. Accordingly, the α 1–3 Gal epitope can be used to identify the production cell line of a mAb in a



Fig. 4.5 (continued) normalized to the sum of all peaks, resulting in a normalized intensity in % of total peak height (TPH). *N*-glycan structures were assigned via database matching using glyXtoolCE™ (in combination with glyXbase™) and confirmed by exoglycosidase sequencing as published by Cajic et al. (Thiesler et al. 2016). Symbolic representation of *N*-glycan structures follows the guidelines of Symbol Nomenclature for Glycans (SNFG) (Varki et al. 2015)

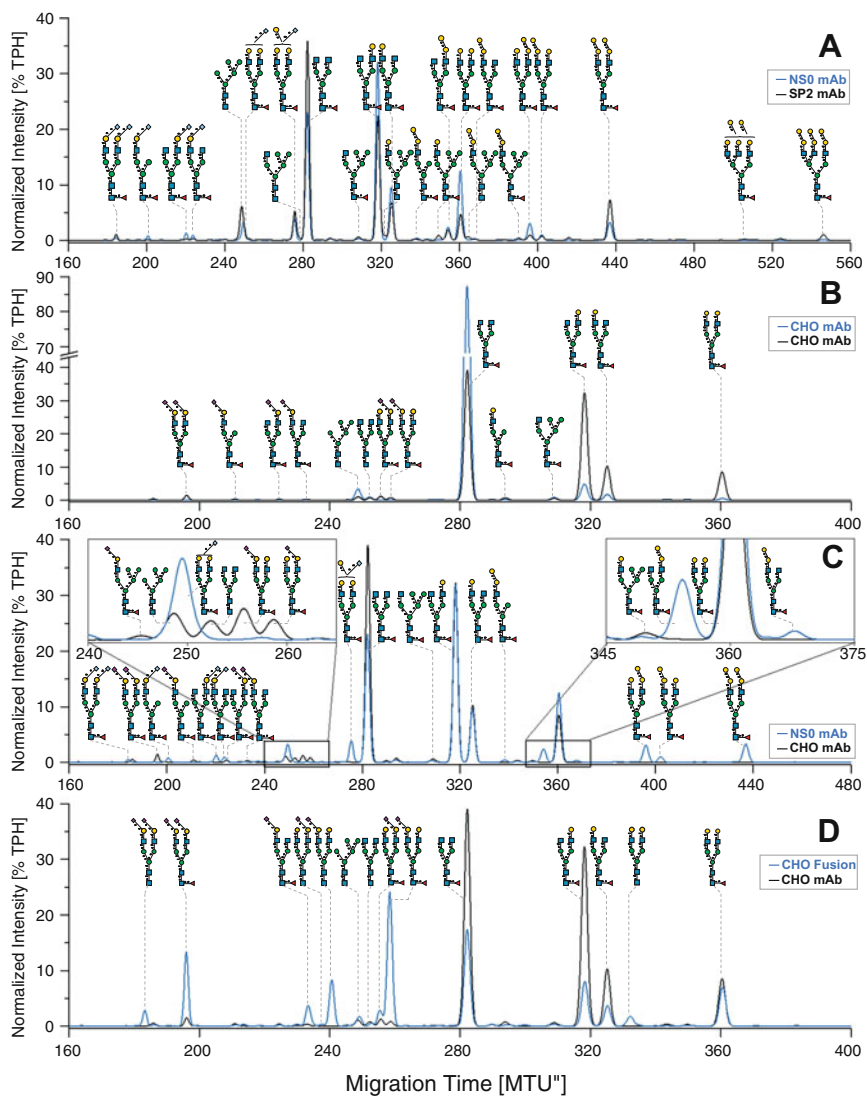


Fig. 4.6 xCGE-LIF-based *N*-glycan analysis comparing the therapeutic proteins ramucirumab (NS0), cetuximab (SP2/0), bevacizumab (CHO), rituximab (CHO) and etanercept (CHO). xCGE-LIF generated fingerprints of APTS-labeled *N*-glycans derived from mAb ramucirumab (Cyramza) produced in NS0 cells (a and c; blue curve), mAb cetuximab (Erbix) produced in SP2/0 cells (a; black curve), mAb bevacizumab (Avastin[®]) produced in CHO cells (b; blue curve), mAb rituximab produced in CHO cells (Rituxan[®]) (b, c and d; black curve) and fusion protein etanercept (Enbrel[®]) produced in CHO cells (d; blue curve) (all purchased at Evidentic). Release and APTS-labeling of *N*-glycans were performed using the glyXprep⁴⁸ kit (glyXera 2020), by carefully following the kit instructions. Data processing was performed using glyXtoolCE[™] (glyXera 2021). Data processing comprised alignment of migration times to two orthogonal internal standards, resulting in a double aligned x-axis in migration time units (MTU^{''}). Peak heights were normalized to the sum of all peaks, resulting in a normalized intensity in % of total peak height (TPH). *N*-glycan structures were assigned via database matching using glyXtoolCE[™] (in combination with glyXbase[™]) and

straightforward manner, as shown for comparison of the CHO-derived mAb rituximab (Rituxan[®]) (black line) and the NS0 derived mAb ramucirumab (blue line) in Fig. 4.6c (zoom-in around 360 MTU''). However, CHO cells are differing from NS0 and SP2/0 cells in two additional important *N*-glycosylation properties: CHO cells incorporate mainly the non-immunogenic Neu5Ac in α 2–3 linkage, as representatively shown for the mAbs bevacizumab (Avastin[®]) (blue line) and rituximab (black line) in Fig. 4.6b and the fusion protein etanercept (Enbrel[®]) (blue line) in Fig. 4.6d (Borza et al. 2018; Mcleod 2013). In contrast, NS0 and SP2/0 cells integrate Neu5Gc in α 2–6 linkage (Qian et al. 2007; Stadlmann et al. 2008; Beck et al. 2008), as shown for SP2/0-derived cetuximab in Fig. 4.5c, d and ramucirumab in Fig. 4.6a. This enables an easy distinction between originator and biosimilar mAb, as both structural *N*-glycan properties (Neu5Gc versus Neu5Ac and α 2–3 versus α 2–6 linkage) can be directly resolved by xCGE-LIF, without the need for an additional exoglycosidase treatment, as shown in detail for the zoom-in around 250 MTU'' in Fig. 4.6c. Furthermore, the high resolution of xCGE-LIF enables the monitoring of structural *N*-glycan properties of glycoengineered CHO cells, like the missing core fucose of FUT8 knockout CHO cells (Yamane-Ohnuki et al. 2004), the additional bisected GlcNAc for GnTIII over-expression CHO cells (Umaña et al. 1999), or the emerging α 2–6-linked Neu5Ac in CHO cells with stable ST6GAL1 expression (Houeix and Cairns 2019).

Besides the resolution of structural properties of *N*-glycans, the relative quantities (like the degree of galactosylation or sialylation) are easily accessible, too. As demonstrated for the mAbs bevacizumab and rituximab in Fig. 4.6b, remarkable differences in *N*-glycosylation can be observed. While bevacizumab has a considerably low degree of galactosylation (with about 90% of all *N*-glycans gathered in only one peak comprising of FA2G0), rituximab shows a more complex *N*-glycosylation pattern (with di-antennary *N*-glycans bearing up to two terminal sialic acids). In the case of sialylation, both mAbs are clearly lagging behind the fusion protein etanercept (tumor necrosis factor receptor linked to the Fc portion of human IgG1) with a sialylation degree of more than 50%. These differences in the *N*-glycosylation are often intentionally induced, as they affect the proper function of the protein (Kanda et al. 2007), its stability and thus the product potency and quality.

Moreover, xCGE-LIF is a powerful tool for high-throughput screening of hundreds of cell clones during early-stage discovery, as well as post-discovery characterization, with minimal material consumption and costs. Here, xCGE-LIF-based methods help to reduce the screening time, greatly accelerating the development of biosimilars, biobetters, and new biotherapeutics. In combination with commercially available enzymes like IdeZ (by New England Biolabs) or FabRICATOR[®] (Z) (by Genovis), "site"-specific characterization of IgG-based therapeutic proteins can be achieved, ensuring a more detailed level of protein characterization, and



Fig. 4.6 (continued) confirmed by exoglycosidase sequencing as published by Cajic et al. (Thiesler et al. 2016). Symbolic representation of *N*-glycan structures follows the guidelines of Symbol Nomenclature for Glycans (SNFG) (Varki et al. 2015)

preventing surprises during pharmacokinetic testing. Consequently, xCGE-LIF holds potential toward detailed analysis of mAb glycosylation in real high throughput.

4.9 Conclusion

CE has matured to the point that it can stay side by side with other more commonly used techniques for glycan analysis. Because of its unbeatable speed, resolution, sensitivity, and simplicity, the method is gaining more and more attention. Ongoing efforts to bring CE-based analysis kits and software solutions on the market (together with the increasing availability, size, and quality of glycan databases) will make the method attractive to the biopharmaceutical industry, helping to reveal the full potential of therapeutic glycoproteins like mAbs. Application of CE-based workflows for Ig analysis will enable more detailed characterization of their glycosylation and fast, automated high-throughput monitoring of their glycosylation patterns, especially when employed for large cohort studies. The recent and upcoming developments in miniaturization and analysis toolboxes show that there is an exciting future in glycan analysis for this recently revived technology.

Compliance with Ethical Standards

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Conflict of Interest Author Erdmann Rapp is the founder, CEO and CSO of glyXera GmbH. Authors René Hennig and Robert Burock are employees of glyXera GmbH. glyXera provides high-performance glycoanalytical products and services and holds several patents for xCGE-LIF based glycoanalysis. Author Samanta Cajic declares no competing interests.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

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