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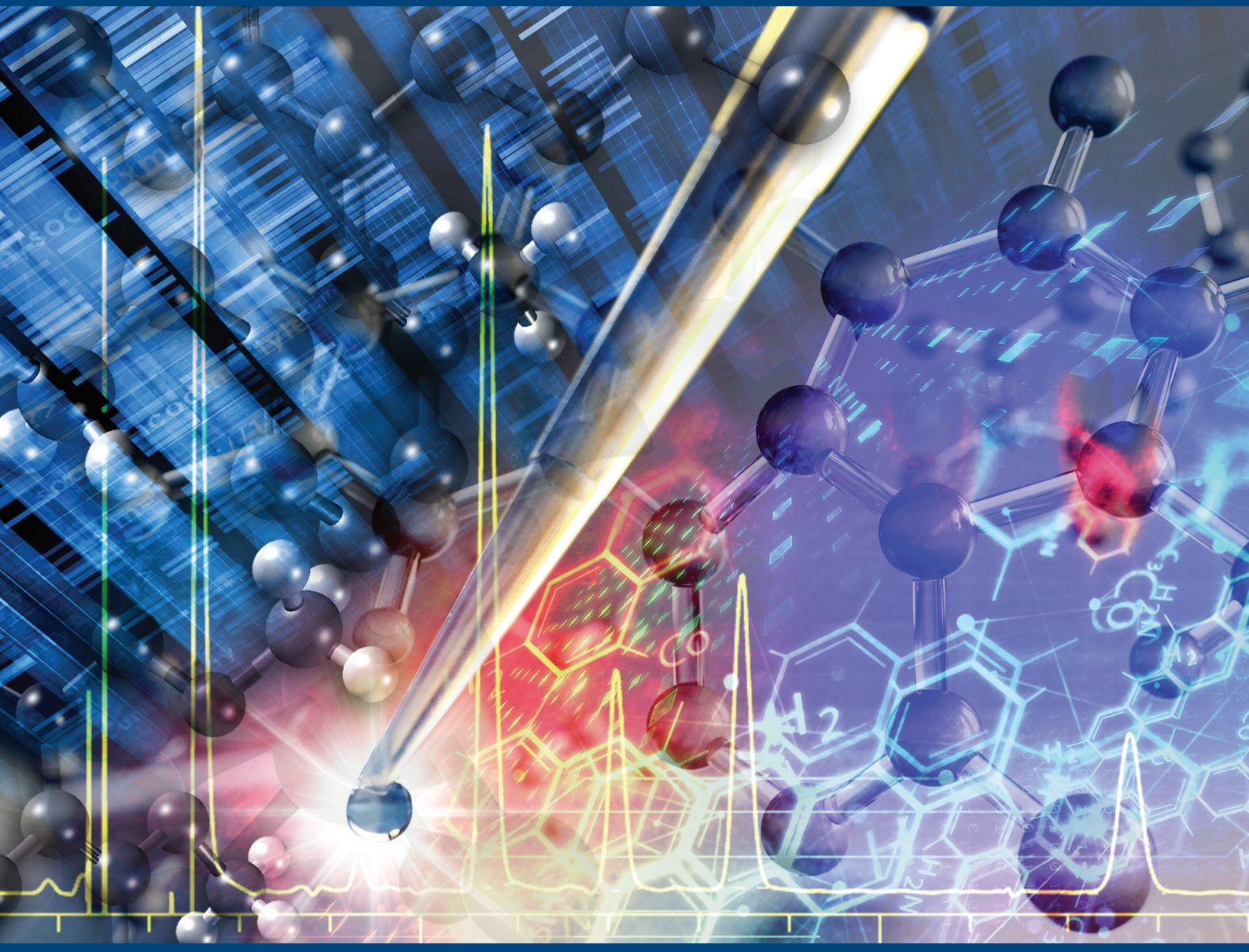

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REVIEW ARTICLE

Therapeutic Fc-fusion proteins: Current analytical strategies

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Fc-Fusion proteins represent a successful class of biopharmaceutical products, with already 13 drugs approved in the European Union and United States as well as three biosimilar versions of etanercept. Fc-Fusion products combine tailored pharmacological properties of biological ligands, together with multiple functions of the fragment crystallizable domain of immunoglobulins. There is a great diversity in terms of possible biological ligands, including the extracellular domains of natural receptors, functionally active peptides, recombinant enzymes, and genetically engineered binding constructs acting as cytokine traps. Due to their highly diverse structures, the analytical characterization of Fc-Fusion proteins is far more complex than that of monoclonal antibodies and requires the use and development of additional product-specific methods over conventional generic/platform methods. This can be explained, for example, by the presence of numerous sialic acids, leading to high diversity in terms of iso-

Article Related Abbreviations: 2-AB, 2-aminobenzamide; 2-DE, two-dimensional gel electrophoresis; 2D-LC, two-dimensional liquid chromatography; actRIIb, activin receptor type IIB; ADCC, antibody-dependent cellular cytotoxicity; AEX, anion exchange chromatography; AF4, asymmetrical flow field flow fractionation; aPTT, activated partial thromboplastin time; AUC, analytical ultra-centrifugation; C1q, complement component 1q; CD4, cluster of differentiation 4; CDC, complement dependent cytotoxicity; CEX, cation exchange chromatography; CHO, chinese hamster ovary; CID, collision-induced dissociation; cIEF, capillary isoelectric focusing; CQA, critically quality attribute; CTLA4, cytotoxic T-lymphocyte-associated antigen 4; CZE, capillary zone electrophoresis; DLS, dynamic light scattering; DMB, 1,2-diamino-4, 5-methylenedioxybenzene dihydrochloride; DTT, dithiothreitol; ECD, electron-capture dissociation; ECD, extracellular domain; EM, electron microscopy; EMA, European medicines agency; ESI, electrospray ionization; ETD, electron-transfer dissociation; EThcD, electron-transfer higher-energy collision dissociation; EU, European union; Fab, antigen-binding fragment; Fc, fragment crystallizable; FcRn, neonatal Fc receptor; FcγR, Fc gamma receptor; FD, fluorescence detection; FDA, U.S. Food and Drug Administration; FIX, factor IX; FVIII, factor VIII; Gla, γ-carboxyglutamate; GlcNAc, N-acetylglucosamine; GLP-1, glucagon-like peptide-1; Glu, glutamate; GU, glucose units; HC, heavy chain; HCD, higher-energy dissociation; HDX-MS, hydrogen–deuterium exchange mass spectrometry; HIC, hydrophobic interaction chromatography; HPAE, high performance anion-exchange; HRMS, high-resolution mass spectrometry; IAA, iodoacetic acid; IAM, iodoacetamide; icIEF, or imaged capillary isoelectric focusing; IC, intended copy; IEF, isoelectric focusing; IEX, ion exchange chromatography; IgG, immunoglobulin G; IL, interleukine; IL-1R, interleukin-1 receptor; IL-1RAcP, interleukin-1 receptor accessory protein; IMER, immobilized enzyme reactor; LFA-3, leucocyte function antigen-3; MALS, multi angle laser light scattering; MES, 2-(N-morpholino)ethanesulfonic acid; MRM, multiple-reaction monitoring; NANA, N-acetylneuraminic acid; NEM, N-methylmaleimide; Neu5Ac, N-acetylneuraminic acid; Neu5Gc, N-glycolylneuraminic acid; NGNA, N-glycolylneuraminic acid; nrCE-SDS, non-reduced capillary electrophoresis sodium dodecyl sulfate; PD, pharmacodynamics; pdFIX, plasma-derived factor IX; pdFVIII, plasma-derived factor VIII; PGC, porous graphitic carbon; PK, pharmacokinetics; PlGF, placental growth factor; PNGase F, peptide-N-glycosidase F; PTM, posttranslational modification; rCE-SDS, reduced capillary electrophoresis sodium dodecyl sulfate; rFIX, recombinant factor IX; rFVIII, recombinant factor VIII; SAXS, small-angle X-ray scattering; SEC, size exclusion chromatography; SPR, surface plasmon resonance; TCEP, tris(2-carboxyethyl)phosphine; TGF-β, transforming growth factor β; TNFR, tumor necrosis factor receptor; TNF-α, tumor necrosis factor α; TNSALP, tissue-nonspecific alkaline phosphatase; TPO, thrombopoietin; USP, United States Pharmacopeia; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; WHO, World Health Organization

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electric points and complex glycosylation profiles including multiple *N*- and *O*-linked glycosylation sites. In this review, we highlight the wide range of analytical strategies used to fully characterize Fc-fusion proteins. We also present case studies on the structural assessment of all commercially available Fc-fusion proteins, based on the features and critical quality attributes of their ligand-binding domains.

KEYWORDS
Fc-fusion proteins, hydrophilic interaction chromatography, ion-exchange chromatography, mass spectrometry, size exclusion chromatography

1 | INTRODUCTION

Peptides and proteins are interesting drug candidates due to their important role in many different disease pathologies. However, the clinical potential of these biomolecules is often hampered by their inherent short serum half-life. As a result of the limited target tissue exposure, many therapeutic peptides and proteins require more frequent dosing intervals to maintain a clinically effective drug concentration [1,2]. This problem brought forth a unique class of therapeutics, namely, Fc-fusion proteins, that combine the beneficial pharmacological properties of biological ligands with the additional properties of the fragment crystallizable (Fc) domain of an immunoglobulin G (IgG). Indeed, fusion of the IgG-Fc domain to a ligand, active peptide or extracellular domain (ECD) of a receptor greatly improves the clinical potential of active protein drugs, for example, by extending the plasma half-life as well as engaging immune-mediated effector functions that may also be silenced [3]. The first Fc-fusion protein was reported in 1989 and comprised the ECD of the cluster of differentiation 4 (CD4) coupled to the Fc fragment of an IgG1 molecule [4]. Less than a decade later, the Fc-fusion protein etanercept (Enbrel®, Amgen) was approved by the U.S. Food and Drug Administration (FDA) for treatment of rheumatoid arthritis. Etanercept combines the ECD of human p75 TNF receptor (TNFR) with a human IgG1 Fc to neutralize both soluble and membrane-bound TNF-α to reduce its inflammatory effects [5]. Currently, etanercept is approved for five additional indications and is one of the top ten best-selling drug products worldwide. However, similar to other Fc-fusion proteins, etanercept is approaching the end of patent-protection in the United States and is yet facing growing competition from biosimilar products approved in Europe [6].

To date, ~37 therapeutic fusion products are in clinical development and thirteen products have been approved by the FDA and the EMA (Table 1). They follow the commercial successes of mAbs with five blockbuster products (aflibercept, etanercept, dulaglutide, abatacept, and efmoroctocog α with 7.5, 7.2, 4.3, 3.2, and 1.2 billion USD sales in 2019, respectively) [7]. Based on these approvals, Fc-fusion proteins are one of the most successful classes of IgG-based products when compared to other classes such as, antibody-drug conjugates, radio-immunoconjugates, or glyco-engineered products (with 8, 2, and 3 products on the market, respectively) [8,9].

Most of the Fc-fusion proteins are produced by genetic fusion of the C-terminus of a biological moiety to the N-terminus of the IgG-Fc domain. The strong interaction of the IgG-CH3 domains creates a stable Fc-structure and allows more complex structures to be fused to the flexible hinge regions [10]. In the hinge region, the disulfide bonds reside at the base of either monomeric, homodimer, and heterodimer structures. As shown in Figure 1, the Fc-fusion partners can be subdivided into four major groups: the extracellular domains (ECD) of natural receptors (e.g., etanercept, belatacept, and abatacept) and novel binding domains, such as, functionally active peptides (e.g., romiplostim, dulaglutide), genetically engineered binding constructs acting as cytokine traps (e.g., aflibercept and conbercept) or recombinant enzymes (e.g., asfotase α and efmoroctocog α).

Generally, these biologically active proteins have very short serum half-lives due to renal clearance and proteolytic metabolism [11]. Renal clearance plays an important role for products with a molecular weight of less than 60 kDa [12]. Therefore, increasing the size of the active protein by coupling to an Fc-domain can bring the construct above the threshold for kidney filtration and increase the circulation time [13]. The added Fc-domain can further prolong the circulation time via interaction with the neonatal Fc receptor (FcRn) in a pH-mediated recycling procedure [14]. Fc-fusion proteins can be protected from lysosomal degradation when they are taken up by endothelial cells and then released back in to the bloodstream by binding of the Fc-fragment to FcRn receptors present in endosomes. Therefore, the target tissue has

TABLE 1 Marketed Fc-fusion proteins. ECD and CFDA stand for extracellular domain and Chinese food and drug administration, respectively

Approval year (US)	Non-proprietary and trade name	Description of protein format	Format in brief	Molecular target
1998	etanercept (Enbrel®)	ECD of the human 75 kDa (p75) tumor necrosis factor receptor (TNFR) fused to human IgG1 Fc	TNFR – Fc fusion protein	TNF-α
2003; withdrawn in 2011	alefacept (Amevive®)	ECD of the human leucocyte function antigen-3 (LFA-3) fused to human IgG1 Fc	LFA3 – Fc fusion protein	CD2-LFA-3 on activated T cells
2005	abatacept (Orencia®)	ECD of human cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) fused to a modified human IgG1 Fc	CTLA4 – Fc fusion protein (modified Fc)	CD80/CD86
2008	rilonacept (Arcalyst®)	Portions of ECD of interleukin-1 receptor (IL-1R) and IL-1R accessory protein fused to human IgG1 Fc	IL1R – Fc fusion protein	Antagonizes IL-1β, IL-1α, IL-1RA
2008	romiplostim (Nplate®)	Polypeptide mimetic sequence of thrombopoietin (TPO) fused to aglycosylated human IgG1 Fc	Peptide – Fc fusion protein (peptibody)	TPO receptor agonist
2011	belatacept (Nulojix®)	ECD of human cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) fused to a modified human IgG1 Fc	CTLA4 – Fc fusion protein	CD80/CD86
2011	aflibercept (Eylea®)	ECDs of human vascular endothelial growth factor (VEGF) receptor 1 (domain 2) and receptor 2 (domain 3) fused to human IgG1 Fc	VEGFR – Fc fusion protein	VEGF
2012	ziv-aflibercept (Zaltrap®)	ECDs of human vascular endothelial growth factor (VEGF) receptor 1 (domain 2) and receptor 2 (domain 3) fused to human IgG1 Fc	VEGFR – Fc fusion protein	VEGF
2013 (by CFDA)	conbercept (Lumitin®)	ECDs of human vascular endothelial growth factor (VEGF) receptor 1 (domain 2) and receptor 2 (domains 3 and 4) fused to human IgG1 Fc	VEGFR – Fc fusion protein	VEGF
2014	efmoroctocog α (Elocta®)	Single molecule of recombinant Factor VIII (rFVIII) fused to human IgG1 Fc	rFVIII – Fc fusion protein	Enzyme substitute of rFVIII
2014	eftrenonacog α (Alprolix®)	Single molecule of recombinant Factor IX (rFIX) fused to human IgG1 Fc	rFIX – Fc fusion protein	Enzyme substitute of rFIX
2015	asfotase α (Strensiq®)	Catalytic domain of tissue-nonspecific alkaline phosphatase (TNSALP) fused to the human IgG1 Fc	Enzyme – Fc fusion protein	Enzyme substitute of TNSPALP
2015	dulaglutide (Trulicity®)	Dipeptidyl peptidase-IV-protected glucagon-like peptide (GLP-1) fused to human IgG4 Fc	Peptide – Fc fusion protein (peptibody)	GLP1R agonist
2019	luspatercept (Reblozyl®)	Modified ECD of activin receptor type IIB (actRIIb) fused to human IgG1 Fc	ActRIIb – Fc fusion protein	Transforming growth factor beta (TGF-β) superfamily ligands

a longer exposure to the pharmacologically active moiety and, consequently, the therapeutic potential of this latter is increased [1,15].

Next to improving the therapeutic potential of the biological partner, the Fc-domain also greatly improves the stability and solubility of, e.g. hydrophobic ligands or receptors [16]. By coupling molecules that are difficult to produce to Fc-domains, higher expression and secretion rates are achieved during production [17]. Production of Fc-fusion proteins is often based on existing antibody manufacturing technology using predominantly mammalian-cell-based processes. Chinese hamster ovary (CHO) cell lines are mostly used for the expression of commercially available Fc-fusion proteins, to ensure the

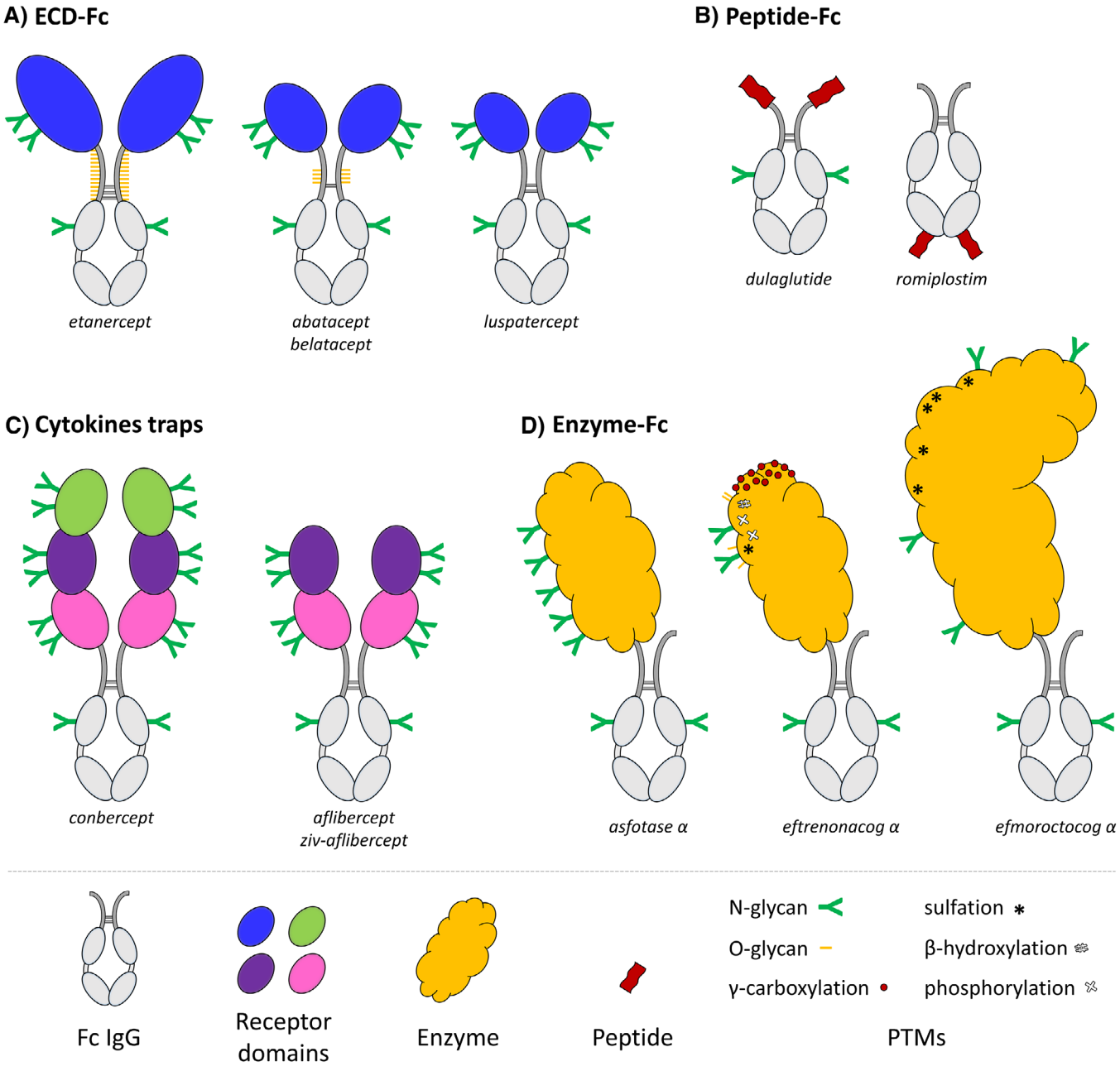

FIGURE 1 Schematic representation of selected Fc-fusion proteins classified based on their ligand binding domain that can be derived from a receptor ECD (A), a peptide (B), a cytokine trap (C), or an enzyme (D). Highlights on the main structural differences linked to the presence of different post-translational modifications (PTMs)

correct conformation and post-translational modifications (PTMs). Unfortunately, CHO cell lines lack the ability to produce the correct glycosylation pattern that resembles the one found on human glycoproteins [18]. Therefore, the CHO cell lines have been genetically modified to express specific human glycosyltransferases during production [19]. Interestingly, efmoroctocog α is produced in an Human Embryonic Kidney cell line (HEK 293) to generate correctly folded and fully active FVIII with human glycans.

Glycosylation is one of the most important PTM due to its major role in the immunogenicity and clinical efficacy of therapeutic proteins [20]. Like most IgG1-4 based mAb products, Fc-fusion proteins contain a canonical N-linked glycosylation site in the CH2 domain, bearing complex bi-antennary glycans with low sialic acid contents [21]. These glycans often have an important effect on the FcγR affinity and they support the folding and stabilization of the Fc-domain by maintaining the favorable "closed" Fc conformation [22]. Compared to mAbs, Fc-fusion proteins

often have multiple N-glycosylation sites and additional *O*-linked glycosylation sites in the biological domain (Figure 1 and Supporting Information). These oligosaccharide structures can have a wider variety of complex bi-, tri-, and tetra-antennary structures [23,24]. This more extensive glycosylation profile can have a significant impact on the in vivo clearance of the Fc-fusion protein via, e.g. the sialic acid content and terminal N-acetylglucosamine (GlcNAc) residues. [25,26] As result of the far-reaching effects of the entire glycosylation profile, it is of utmost importance to perform comprehensive and site-specific glycan analysis as part of the quality control strategy of Fc-fusion protein [27].

Next to glycosylation, Fc-fusion proteins are prone to many other PTMs that result in size, charge, and other product variants that can hamper the product efficacy and safety. One of such PTMs is the oxidation of methionine residues in the CH_2-CH_3 domain that can result in the reduction of FcRn binding and cause a significant reduction in the serum half-life of IgG [28–30]. The combination of the inherent microvariability of mAbs (e.g. Fc-glycosylation and PTMs) with the added variability of the fused partner, creates structurally complex products. Therefore, the comprehensive characterization of Fc-fusion proteins relies on highly specific analytical and bioanalytical strategies in order to keep up with the increasing complexity of Fc-fusion proteins. In this review, we will discuss the most recent analytical strategies that are used to gain a better understanding of the critical product characteristics and ensure safe and efficacious products on the market. In addition, several case studies will be presented to illustrate different analytical approaches applied for the characterization of a wide variety of Fc-fusion protein formats and the challenges accompanied with the emerging biosimilar market for Fc-fusion proteins will be critically discussed.

2 | ANALYTICAL METHODS FOR Fc-FUSION PROTEINS CHARACTERIZATION

The structural complexity and heterogeneity of Fc-fusion proteins (Figure 1 and Supporting Information) requires a set of analytical tools in order to be properly characterized. This section describes the analytical methods used to confirm the primary structure of the Fc-fusion proteins (Section 2.1), to evaluate the main PTMs, such as charge/size variants and glycan profile, and the main physicochemical properties of Fc-fusion proteins, such as identity, purity, and integrity. The identity of an Fc-fusion protein can be determined by a variety of analytical methods including ion exchange chromatography (IEX), imaged capillary isoelectric focusing (icIEF), hydrophobic interaction chromatography (HIC), RP-LC, and peptide mapping using LC–MS [31–33]. Among them, IEX and icIEF are mostly used because the distribution of charge variants (Section 2.2) of an Fc-fusion protein provides a distinctive fingerprint of the protein. Purity and integrity confirmation of Fc-fusion proteins is required throughout all stages of manufacturing, storage, and administration to the patient. Like other therapeutic proteins, Fc-fusion proteins are susceptible to PTMs and degradation that could eventually result in fragmentation and aggregate formation. The purity assessment and size variant (Section 2.3) characterization are traditionally performed by size exclusion chromatography (SEC) or CE sodium dodecyl sulfate (CE-SDS), by measuring the level of high molecular weight species, monomer, and fragments such as target peptide-Fc variants [34–36]. At last, methods used for the glycan analysis by hydrophilic interaction chromatography (HILIC) will be discussed in more detail in Section 2.4. [37,38]

2.1 | Confirmation of primary structure by MS analysis

For the confirmation of the primary structure of Fc-fusion proteins by MS, a large number of analytical techniques is applied at different analyte levels (intact, middle-up/down, and bottom-up/down) that together provide the full and comprehensive characterization of the Fc-fusion protein.

Intact protein mass measurement is a simple and fast method that can both reveal the heterogeneity of therapeutic proteins and allow comparing molecular mass [39,40]. Similarly to mAbs, an array of separation techniques such as LC and CE can be applied for Fc-fusion protein characterization. However, due to the high heterogeneity of Fc-fusion proteins, especially caused by the presence of multiple *N*- and *O*-glycosylation sites, there are some challenges that may require analytical strategies to reduce complexity at the intact level [26]. For instance, optional deglycosylations by PNGase-F to specifically remove N-glycans or eventually sialidase and O-glycosidase for the O-glycans, are commonly reported. [40,41] Figure 2 shows an example of deconvoluted ESI mass spectra of an intact dimeric Fc-fusion protein after reduction and de-*N*-glycosylation performed for comparability studies.

Middle-up strategy based on LC–MS analysis of subunits generated after specific enzymatic digestion in the hinge region of the HC, yielding Fab or $(Fab')_2$ and Fc fragments, is gaining more interest due to its complementarity to the intact mass and bottom-up analysis [42–44]. The minimized sample manipulation, which is usually performed within 1 h, provides a significant time saving and reduces the risk of potential artifacts [45]. This approach has been successfully applied for the elucidation of molecular

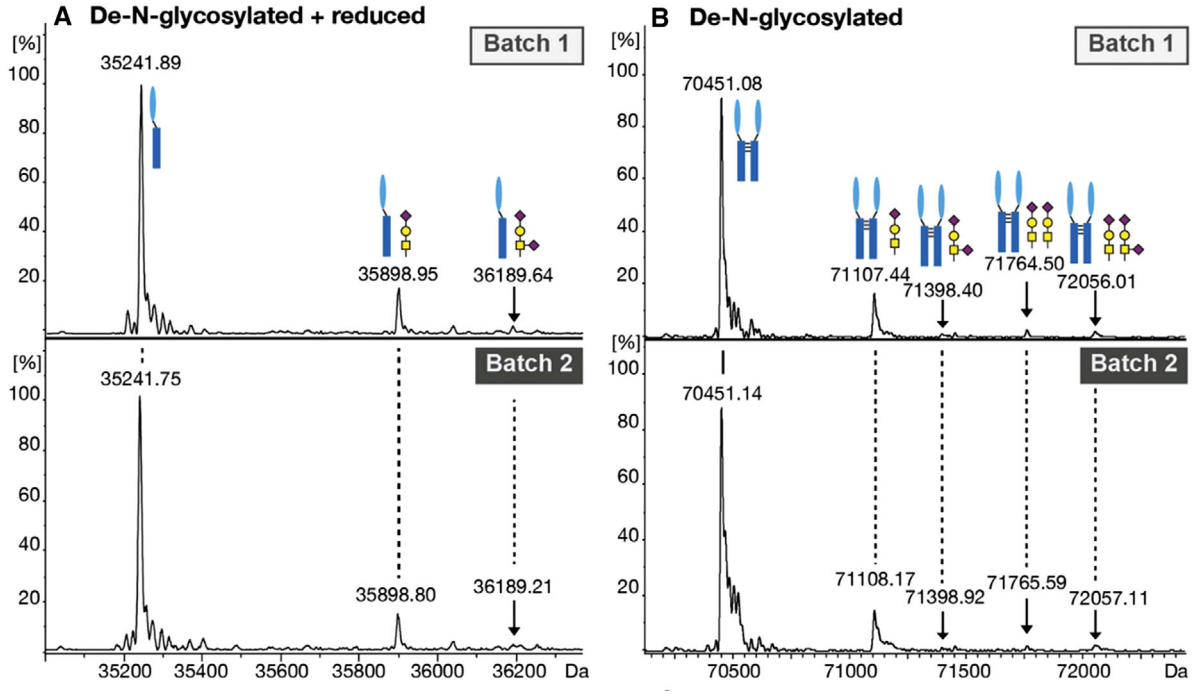

FIGURE 2 Intact protein mass measurement of O-glycosylation variants of a Fc-fusion protein by CE-MS. (A) Deconvoluted spectra of the monomeric reduced de-N-glycosylated protein species. (B) Deconvoluted spectra of the dimeric de-N-glycosylated protein species. Deconvoluted spectra of the dimeric de-N-glycosylated protein species for two batches. Reprinted with permission from [41]

properties such as disulfide linkages, combination of multiple modifications, and glycosylation profiling of the Fc/2 subunit [46–50].

The middle-up approach is particularly interesting for Fc-fusion proteins, which usually contain a biological target attached to an immunoglobulin Fc region through its N-terminal or C-terminal. Different proteases have been used for the digestion of Fc-fusion proteins, including papain, pepsin, Lys-C, and more recently, *IdeS* [51–54]. For instance, Kleemann et al. reported a study on limited proteolysis using endoproteinase Lys-C to localize oxidation sites, since the active protein parts were attached either to the N- or C-terminus of the Fc fragment with a Lys residue. [54] The resulting fragments, the Fc domain fragment and the individual peptide moieties including the oxidized peptides, were successfully separated by a RPLC–MS, which made the quantitation of oxidation possible. In another study, Yu et al. reported an LC–MS/MS method for characterizing degradation of Met1 and Asp2 residues at the N-terminus of the Fc region, by employing a limited endoproteinase Glu-C digestion [52]. A dimeric peptide was generated by limited digestion and it was identified and quantified together with its modifications including Met1 oxidation and Asp2 isomerization, which are usually not detectable following trypsin and Lys-C proteolysis. Middle-down approach, which refers to MS/MS-based experiments, can be also performed for sequencing of fragments [55,56]. Regl et al. described a middle-down approach employing *IdeS* under reducing conditions for the quantification of oxidation in the Fc portion of IgG1 mAbs as well as Fc-fusion proteins [56]. The authors reported limits of detection of 1.2, 1.0, and 1.2% for oxidation level in drug products containing the biopharmaceuticals rituximab, adalimumab, and etanercept, respectively. More recently, D'Atri et al. described an effective middle-up approach using HILIC combined with HRMS to assess glycosylation of etanercept [53]. The authors used the *IdeS* protease to cleave etanercept at the hinge region to yield two Fc/2 fragments and the dimeric TNFR subunit. Beyond the use of *IdeS* protease, additional specific enzymes would be beneficial for the characterization of certain Fc-fusion protein. For instance, *IdeZ* enzyme can be suitable for the Fc-fusion proteins containing the LALA mutation, such as dulaglutide (see Supporting Information).

Bottom-up analysis (peptide mapping) performed by LC–MS/MS is the gold-standard technique for the confirmation of amino acid sequences, as well as the identification and quantification of protein variants and site-specific modifications of Fc-fusion proteins [39]. PTMs such as methionine or tryptophan oxidation, asparagine deamidation, aspartic isomerization, lysine glycation, glycosylation, and N- and C-terminal variants, can be simultaneously characterized in a single LC–MS/MS analysis [45]. The peptide mapping approach involves multiple sample preparation steps such as (i) reduction of the protein; (ii) alkylation of Cys residues; and (iii) enzymatic digestion

with trypsin or other endoproteinases (e.g. Lys-C, Asp-N, chymotrypsin, or Glu-C), to cleave the protein into a reproducible set of peptide fragments suitable for analysis by LC–MS/MS. The trypsin digestion is commonly performed under slightly basic pH conditions to ensure optimal digestion efficiency. However, at basic pH conditions potential artifacts such as deamidation or isomerization can occur and result in a possible overestimation of those modifications [57]. A number of studies have suggested adapted protocols to circumvent this issue. Lowering the pH during the enzymatic digestion has proved to be an effective solution to reduce artifacts due to sample preparation [58]. In another paper, Ren et al. focused on reducing the trypsin digestion time to 30 min by optimizing the enzyme activity by removing the guanidine from the digestion buffer [59]. The throughput of trypsin digestion improved significantly compared with conventional trypsin digestion protocols (30 min vs. overnight). On the other hand, the use of immobilized enzyme cartridges has been investigated [60–62]. Recently, Perchepied et al. described the development of two immobilized enzyme reactors (IMERs) based on trypsin and pepsin proteases for the specific mapping of the N-glycosylation heterogeneity of glycoproteins [60]. The authors reported on the complementarity of the trypsin and pepsin protease for the glycosylation mapping, where the pepsin digestion at acidic pH enabled an increase of the sequence coverage, without producing artifacts. Finally yet importantly, the sample preparation steps were completely automated to facilitate multiple characterization. A number of studies highlighted the potential of online peptide mapping workflows, which require significantly lower sample amount and provide faster turnaround times compared to standard approaches with manual sample preparation [61,62]. In this context, comparison of off-line (i.e. manual sample preparation) versus online peptide mapping approaches for the PTM characterization showed overall similar levels of oxidation and deamidation. Lower amounts of deamidation and isomerization were observed at some residues with the online approach, suggesting that there was a reduction in digestion-related artefacts when using the online methodology [62].

Peptide mapping has been successfully implemented for the characterization of Fc-fusion proteins, providing detailed information on glycans and glycopeptides, including the characterization of *N*- and *O*-glycosylation modifications [24,40,41,45]. The use of alternative fragmentation techniques in HRMS such as electron-capture dissociation (ECD), electron-transfer dissociation (ETD), and electron-transfer higher-energy collision dissociation (EThcD) are particularly valuable when combined with commonly applied collisional activating techniques (including collision-induced dissociation (CID) and higher-energy dissociation (HCD)) [62,63]. Typically, these fragmentation techniques can preserve the fragile glycosidic linkage with a low energy fragmentation process, and at the same time, the amino acid residue and the glycosylation sites at the peptide level can be determined [41]. This method can be particularly useful for site-specific *O*-glycosylation analysis, as no consensus sequence for mucin-type *O*-glycosylations is known, and *O*-glycopeptides often contain multiple or continuous glycosylation sites [24]. For instance, EThcD has been used for site-specific *O*-glycosylation analysis of commercially available GLP1-Fc fusion protein with $(G4S)_3$ linker peptide, enabling the detection of unexpected *O*-xylosylations in the $(G4S)_3$ linker and mucin-type *O*-glycosylations in the GLP-1 peptide [64]. Based on these studies, peptide mapping combined with multiple collisional dissociation techniques is a suitable tool for the comparison of biosimilar and innovator product glycosylation heterogeneities. In addition, Huang et al. reported how multiple fragmentations including CID and ETD can be implemented to characterize disulfide linkages, including the TNFR portion (22 disulfide bonds) of two etanercept products (Enbrel® and TuNEX®) [65].

For more information on the cleavage specificity of different enzymes, Bobaly et al. described protocols to perform tryptic proteolysis, *IdeS* and papain digestion, reduction as well as deglycosylation by PNGase F and EndoS2 enzymes and overviewed general sample preparation approaches used to attain peptide, subunit, and glycan level analysis [66,67].

2.2 | Charge variants

Many of the possible modifications of an Fc-fusion protein can lead to changes in surface-exposed charged residues or in the overall change of the surface charge distribution [68,69]. These modifications (variants) are mostly referred to as charge variants. Compared to the main isoform, species with a lower apparent isoelectric point (p*I*) are considered as acidic species, while species with higher p*I* values referred to as basic variants.

The most common modifications resulting in charge variants are deamidation, isomerization, C-terminal lysine variations, N-terminal cyclization, and most importantly sialylation [70]. Most Fc-fusion proteins contain both N-linked and O-linked glycosylation sites. All *N*-linked sites and multiple *O*-linked sites are occupied with typical mammalian oligosaccharide structures. These sugar structures are partially terminated with negatively charged sialic acid residues (sialylation). Different amounts of sialic acid capping of oligosaccharides, attached to different sites on the protein, can lead to a very complex mixture

of isoforms [71]. Loss of protein sialylation could lead to a low final sialylation level and bring negative effects on subsequent clinical efficacy [72]. Sialylation affects half-life, charge distribution (and more generally the p*I*), and other biochemical properties of therapeutic glycoproteins. In general, Fc-fusion proteins possess a high degree of glycosylation and complex sialylation patterns that make it difficult to analyze charge variants at intact level.

Despite the difficulties related to the inherent heterogeneity of Fc-fusion proteins, the regulatory authorities require the deep characterization and quality control of charge variants in biopharmaceuticals to demonstrate similarity of the drug substance between manufactured batches, throughout the production continuum [73]. The most commonly applied analytical methods for charge variant analysis of therapeutic proteins are IEX and electrophoretic techniques such as IEF or icIEF.

Both anion- (AEX) and cation exchange (CEX) mode can be used depending on the character of the Fc-fusion protein. CEX is preferred if p$I \geq 7$ (basic Fc-fusion proteins), while for proteins possessing apparent p$I \leq 7$ (acidic Fc-fusion proteins), AEX is the appropriate mode. IEX chromatography can be run in three different elution modes: (1) salt gradient, (2) pH gradient, and (3) the combination of the two methods, which is often referred as salt-mediated pH gradient [39]. The latter elution mode merges the benefits of the salt- and pH gradients, namely that proteins will elute more or less in the order of their p*I* and will elute in sharp peaks thanks to peak focusing effect of the increasing ionic strength [74]. Because of the non-volatile nature of the salts (NaCl, KCl) and buffers (MES, phosphate) commonly used in IEX mobile phases, IEX is inherently not compatible with MS detection. To solve this problem, a series of trap cartridges can be applied for desalting the IEX effluent before entering the MS system [75]. Another possibility is to replace common salts and buffer components with MS-compatible buffers. Currently, ammonium acetate and ammonium carbonate (or bicarbonate) buffer systems are used and seem to be promising in providing appropriate chromatographic retention and peak shape, together with suitable MS signals [76]. Another possibility to make IEX chromatography compatible with MS, is to use 2D setups, by adding an MS compatible chromatographic mode (e.g. reversed phase) in the second dimension. The other advantage of the 2D setup is that peak capacity and thus separation power can be significantly improved compared to a unidimensional IEX separation, which is a key benefit for highly heterogeneous Fc-fusion proteins [77].

Because of the high resolving power and available miniaturized format, CE is also widely applied for the analysis of the charge variants of biopharmaceuticals. In the different electrophoretic modes, a high electrical field is applied to separate molecules based on differences in charge, size, or hydrophobic properties [39]. CZE is a technique adapted for the separation of proteins with modifications or degradations that affect the charge of the molecules. Next to CZE, capillary isoelectric focusing (cIEF) is a reference technique and complementary/supplementary method to pH gradient IEX. cIEF enables the separation of proteins based on their p*I*, in a pH gradient generated between the cathode and anode, by using ampholytes mixed with the sample to establish the pH gradient. When protein zones are focused into highly concentrated bands at their p*I*, precipitation may occur. It can cause capillary clogging or irreproducible results. To avoid such difficulties, urea, sucrose or a mixture of both can be added to the sample to improve solubility. The mobilization step tends to broaden peaks and increases analysis time, therefore icIEF can be used to allow performing faster separations, higher resolution, as well as better reproducibility, thanks to the whole column imaging technology within a transparent capillary. In icIEF, the separation is performed in a short capillary (5 cm long, 100 μm inner diameter silica capillary) and the IEF process is monitored online by the whole-column detection system at 280 nm wavelength. The advantage of this technique over conventional cIEF is that mobilization of the protein zones is not required; therefore the charged variants of a protein sample can be simultaneously recorded by the whole-column detector without disturbing the separation resolution [32].

Generally, the main purpose of charge variant analysis is to determine (estimate) the relative amount of acidic and basic variants of a protein sample compared to the main isoform and to identify the main variants. In addition, the p*I* of the species can also be estimated and some specific modifications (e.g. lysine truncation, N-terminal variability) can be monitored too. However, it is important to mention that different analytical techniques may give different results [31]. IEX and icIEF techniques are complementary tools and thus should be applied together to obtain as much information about charge variants as possible.

It worth mentioning that due to the huge variability of Fc-fusion proteins (see Figure 1 and Supporting Information), as their size, shape, surface charges, glycosylation level, sialylation level, apparent p*I* that can be drastically different, it is hardly possible to develop platform or generic methods. In contrast, for mAbs (even if they belong to different subclasses) generic cation exchange chromatographic methods are routinely used. Unfortunately, such approach is hardly feasible for Fc-fusion proteins, the methods must be optimized individually.

Navas et al. recently illustrated the difficulties related to the charge variant analysis of ziv-aflibercept and performed a statistical design of experiments to optimize

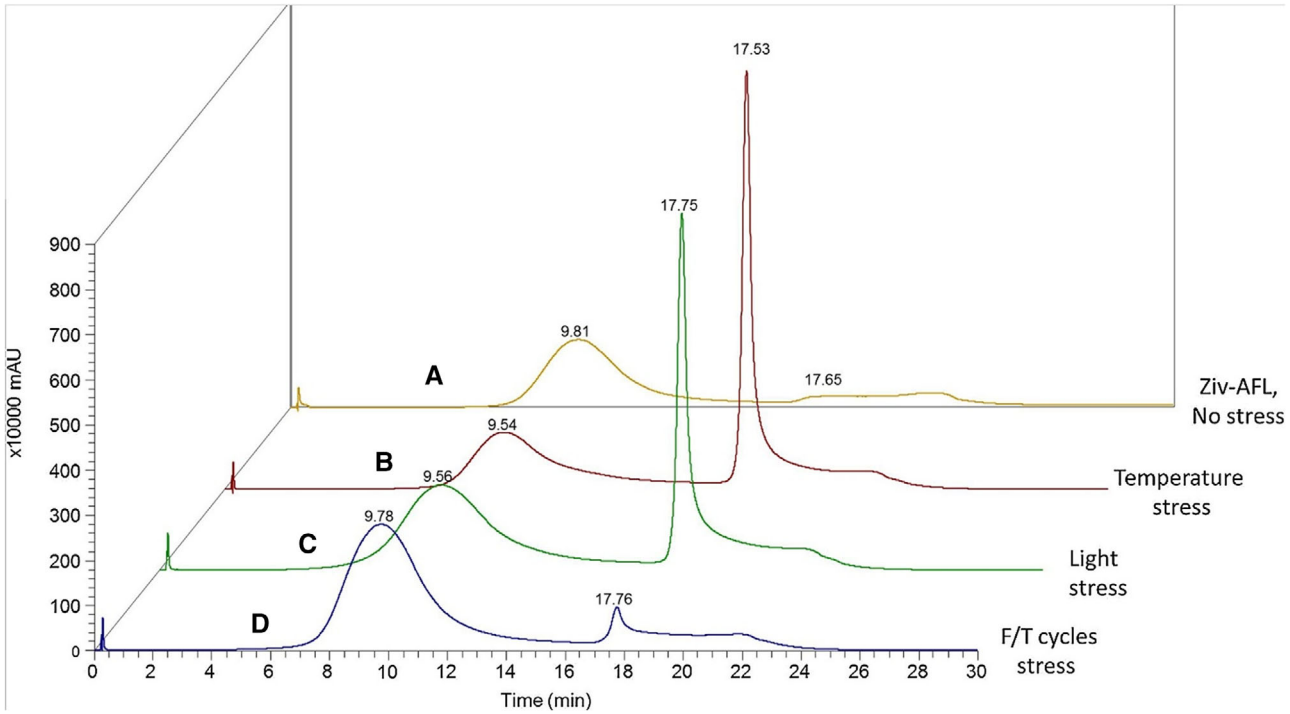

FIGURE 3 CEX chromatograms of controlled degradation study samples (Ziv-aflibercept) using non-volatile buffer: (A) fresh/control sample; (B) ziv-AFL heat stress 60°C; (C) ziv-AFL light stress; and (D) ziv-AFL F/T cycles stress. Reprinted with permission from [78]

the separations using either volatile or non-volatile salts in CEX [78]. Ziv-aflibercept is a complex fusion protein, which is used for the treatment of colorectal metastatic cancer. Two CEX methods were compared: one using pH gradient mode with volatile, low ionic strength buffers and coupled to MS, while the other one was a salt-mediated pH gradient with non-volatile, high ionic strength buffers. The classical method with non-volatile salts in the mobile phase (not MS compatible) performed better in overall separation of charge variants than the MS compatible method (using volatile salts). The MS-compatible method resulted in a single broad chromatographic peak and too complex mass spectra. It was impossible to identify the charge variant species. The reason was probably that ziv-aflibercept is a highly glycosylated fusion protein (15% of the total mass) with a high proportion of sialic acid. This produces numerous states of charges which complicate the mass spectra. At the end of the study, the authors concluded that CEX-MS analysis of intact fusion proteins is quite limited and thus of low importance. The results suggested that conventional CEX methods (with non-volatile salts in the mobile phase) could be better used to characterize and track changes of the charge variant profile, even though it could not be coupled to MS. The increase of basic protein degraded compounds was the most important degradation pattern detected in the stressed samples. Figure 3 shows some representative chromatograms obtained with non-volatile mobile phase by CEX.

As illustrated, intact fusion protein charge variant analysis remains quite limited. Therefore, to reduce the complexity of the sample, sialic acids are often removed (desialylation) from the protein prior to analysis (by using sialidase enzymes or by chemical reagents) [53]. It is important to mention that sialic acids might also have an impact on apparent p*I* and charge variants evaluation. Tan et al. demonstrated for recombinant human TNFR-Fc (Enbrel®) that despite the protein backbone has a theoretical p*I* of 7.2, they experimentally measured p*I* between 4.2 and 6.0 for multiple variants [79]. Partial release of sialic acid resulted in a gradual shift towards higher p*I*. After extended desialylation, only two major and four minor species were focused (IEF) around p*I* 7.2, while without desialylation a much more complex charge profile was obtained. Analysis of the neuraminidase treated samples for residual sialic acid indicated a loss of four sialic acids after a mild sialidase treatment and complete desialylation after excessive enzyme treatment. This indicated that the complexity of the TNFR-Fc preparation is mostly due to heterogeneity in sialic acids attached to the oligosaccharide residues.

To improve the separation power of charge variant analysis, multidimensional setups can be promising tools. Charge heterogeneity and isoform pattern of abatacept was studied by applying a 2D gel electrophoresis (2D electrophoresis) setup [80]. In order to achieve efficient separation of such a complex analyte, 2D electrophoresis

was optimized by employing different experimental conditions regarding the selection of an immobilized pH gradient, sample pretreatment, and detection procedure. It was found that this 2D setup was a suitable tool for the assessment of identity, purity, structural integrity, isoform pattern, and to monitor charge heterogeneity and post-translational glycosylation of the Fc-fusion protein abatacept.

To conclude, the charge variant analysis of Fc-fusion proteins remains very limited at an intact level even with MS detection. Desialylation and/or partial digestion (fragment or subunit analysis) can significantly decrease the complexity of charge variant analysis. Multidimensional separations are probably beneficial however only a very few publications are available so far. Finally, it seems that developing platform methods is hardly possible, individually optimized methods are required.

2.3 | Size variants

In addition to charge and glycosylation variants, size variants are considered as a critical quality attribute (CQA) by the regulatory agencies. Fragment characterization, and more importantly, aggregate characterization is a major concern due to their immunogenic properties. Several complementary analytical techniques are required to cover the wide size range and various physicochemical properties of the size variants during their characterization. As reported by den Engelsman et al., these techniques rely on different separation and detection principles with their own pros and cons [81]. Basic approaches such as visual and microscopic inspection can be used for large aggregates and more complex techniques such as MS for smaller aggregates [36,82]. When information about structural modifications are desired, spectroscopic methods may also be required [83]. Table 2 provides a summary of the techniques used for size variants analysis according to their size range targets. Among the plethora of methods, SEC and CE-SDS are often used as initial screening methods in QC environment, for the analysis of aggregates and fragments, respectively [82]. Both techniques have the main advantage to be robust and high-throughput separation techniques for the separation of size variants and can be hyphenated online to different detectors [81].

According to monograph 129 of the United States Pharmacopeia (USP), SEC is the gold standard method for aggregates characterization and quantification and, to a lesser extent, fragmentation of IgG isotype mAbs [81,84]. As Fc-fusion proteins share a lot of similarity with IgG mAbs, the majority of them can be characterized in the same way [85].

TABLE 2 Summary of the techniques used for size variants analysis. Adapted from [79], with permission

Method	Size range
Methods for detection and characterization of visible and subvisible particles	
Visual inspection	>50 μm-mm
Optical microscopy	>1 μm-mm
Light obscuration	2-100 μm
Flow imaging	1-400 μm
Fluorescence microscopy	>1 μm-mm
DLS	1 Nm- 5 μm
MALS	kDa-MDA range
Turbidity	N/A
Separation techniques for detection and characterization of protein aggregates	
SEC	1-50 nm
SDS-PAGE	kDa – MDa
Native PAGE	kDa – MDa
CE-SDS	kDa – MDa
AF4	1 nm – few μm
Electron microscopy	Nm-mm
Atomic force microscopy	Nm range
"Native" MS	Atomic resolution up to MDa range
Macro-ion mobility spectrometry	3–65 nm or 5 kDa – 100 MDa
AUC	1 nm – 0.1 μm
Indirect methods for assessing protein folding states	
Infrared spectroscopy	N/A
Raman spectroscopy	N/A
UV-VIS spectroscopy	N/A
Fluorescence spectroscopy	N/A
Circular dichroism spectroscopy	N/A

Proteins are separated in SEC according to their hydrodynamic radii on a stationary phase constituted of spherical porous beads with a carefully controlled pore size. These biomolecules diffuse based on their molecular size through the column using an aqueous buffer as the mobile phase. Large aggregates are unable to enter the pores, while smaller molecules can. Since the passage through the pores is longer for smaller molecules, the elution time will increase with the reduction of the hydrodynamic radius [81]. However, SEC can only give an estimation of the molecular weight of the aggregates because the hydrodynamic volume of the molecule does not accurately relate to the molecular weight [83]. To correlate the hydrodynamic radius with the molecular weight, the shape of the protein should be spherical, which is not the case [81,86]. Furthermore, low-affinity aggregates can remain

undetected by SEC, due to the risk of their dissociation in SEC conditions. Additionally, the particle size range is limited by the pore distribution of the stationary phase, system frits, and column inlet. This potentially results in the exclusion of large size aggregates prior to detection [81].

Ideally, in SEC there is no interaction between the biomolecules and the stationary phase. In reality, nonspecific interactions do occur between the proteins and the charges or the hydrophobic surface sites of the stationary phase, resulting in hydrophobic or electrostatic interactions [34].

These undesirable interactions can negatively affect resolution and peak shape, limiting the chromatographic accuracy of SEC, and may result in increased elution time, and therefore leading to an underestimation of the molecular weight [86]. To overcome this issue, organic modifiers can be added to the mobile phase to limit hydrophobic interactions or the ionic strength of the eluent can be increased to limit electrostatic interactions [87]. Therefore, SEC is commonly performed using a high concentration of non-volatile salts in the mobile phase, making this technique inherently incompatible with MS as these salts will lead to ion suppression and contamination of the MS instruments. However, hyphenation of SEC with MS could be beneficial for providing accurate molecular weight information [81]. Therefore, several analytical strategies have been implemented to deal with the non-volatile salts. One of these consists in performing off-line MS detection with matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) analyzer. Otherwise, hyphenation of SEC to MS can be accomplished by 2D-LC, with SEC in the first dimension and RPLC in the second dimension, to remove the non-volatile salts prior to MS detection [88]. Nevertheless, in all the above-mentioned methods, the hyphenation with MS leads to dispersion of the non-covalently associated aggregates. Indeed, the major advantage of SEC is that the conventional methods are performed under non-denaturing conditions, preserving both covalent and non-covalent aggregates. To prevent the loss of information, native ESI-MS methods were developed. For this, native elution conditions are required to allow online hyphenation of SEC and MS, while preserving non-covalent size variants [89]. SEC-MS methods using volatile mobile phases (ammonium acetate) was developed by Goyon et al. for several therapeutic protein formats. This study demonstrated that secondary electrostatic interactions might have a crucial impact on the suitable baseline resolution between HMWS and the main chromatographic peak. Indeed, the use of volatile salts in the mobile phase turned out to be restricted to acidic therapeutic antibodies (pI < 7), while non-volatile salts (potassium phosphate buffer and KCl) were always required when analyzing basic antibody products (pI > 7) [90]. However, the hyphenation of SEC with MS for Fc-fusion proteins could be more straightforward than for other formats. Indeed, due their overall acidity conferred by the sialic acid content, Fc-fusion proteins were less prone to unwanted ionic interaction and therefore successfully analyzed with MS-compatible mobile phases (Figure 4).

CE-SDS is also routinely used in QC environments, but unlike SEC, this technique is commonly used for fragments analysis. CE-SDS is an adaptation of SDS-PAGE in a capillary format [91]. The same principle of molecular sieve is kept, but the slab gel used in SDS-PAGE is converted in a soluble polymer that is filled in a CE-SDS capillary. This technique has numerous advantages over the traditional SDS-PAGE, such as easier automation, better reproducibility and robustness, higher resolution, and speed [35,91,92]. Furthermore, the throughput of CE-SDS has been further increased with the arrival of turnkey instruments, such as the Maurice™ developed by Protein simple (San Jose, CA, USA), for which generic and automated sample preparation methods are supplied [35].

Prior to analysis, samples are heated in the presence of SDS to denature the secondary and tertiary structure of the protein and allow the SDS to coat the entire molecule [35]. Proteins are bound to SDS according to a constant binding ratio, resulting in uniformly charged proteins. Since the capillary is filled with the soluble polymer that acts like a sieving matrix, the proteins are electrophoretically separated based solely on their hydrodynamic radius. Then, the separated proteins are usually identified by UV detection [35,36,82]. It should be noted that non-covalent aggregates are not detected by CE-SDS, because SDS dissociates aggregates constituted by non-covalent interactions [35,81].

Non-reduced (*nr*CE-SDS) and reduced CE-SDS (*r*CE-SDS) can be performed to study size heterogeneity of Fc-fusion proteins. Discrimination between aggregates formed by disulfide bonds and those held together by other covalent bonds could be accomplished by *r*CE-SDS. [35,81] This is not the case in *nr*CE-SDS, because alkylating agents, such as iodoacetamide (IAM), iodoacetic acid (IAA) or N-methylmaleimide (NEM), are used to block free thiols from forming disulfide bonds [36].

One of the weaknesses of CE-SDS is the possible introduction of artifacts during the sample preparation due to reducing agent, heating time, and temperature. IAM used in *nr*CE-SDS may also lead to artifact fragmentation to due insufficient alkylation and protein interaction with the capillary could occur and hamper the aggregate quantification. [35] In addition, CE-SDS is considered as less sensitive than SEC because in this latter both covalent and non-covalent aggregates can be detected [81].

However, it should be noted that both SEC and CE-SDS are not sufficient to cover all types of protein aggregates

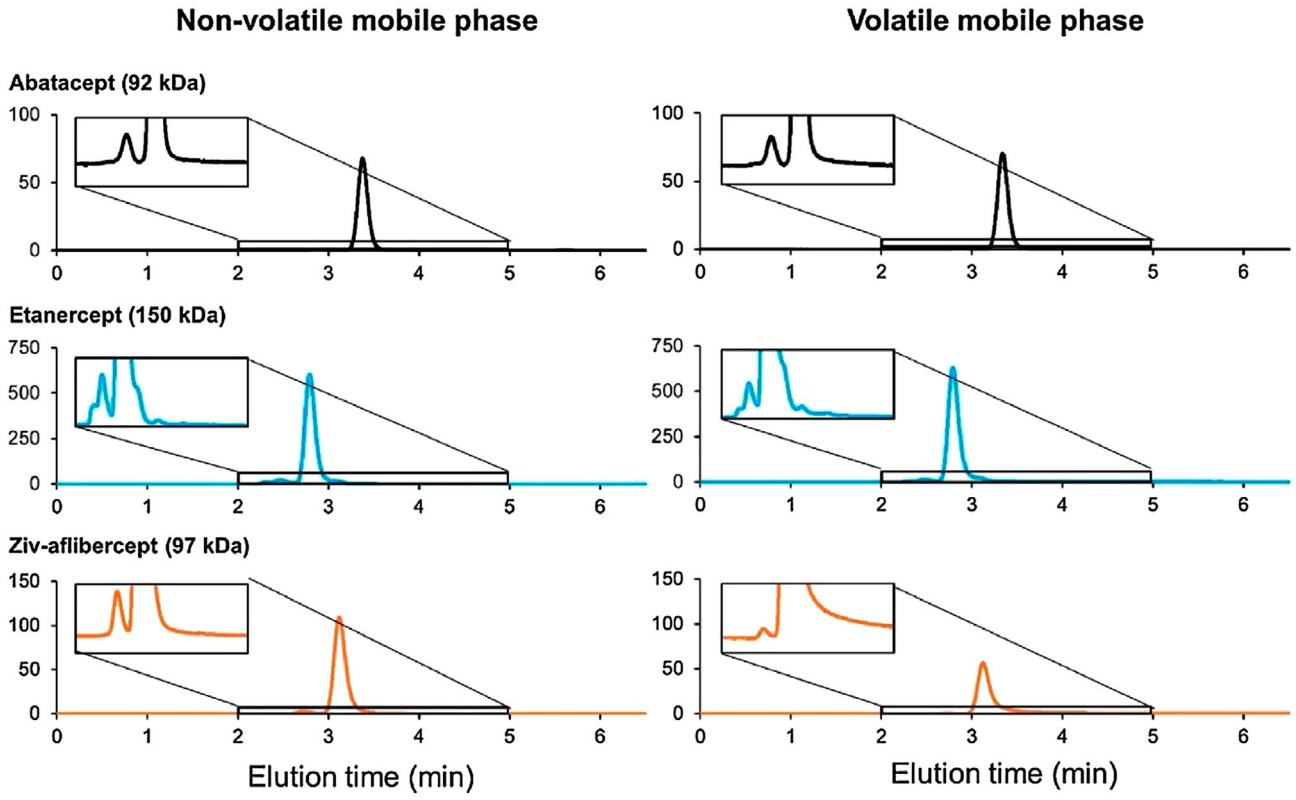

FIGURE 4 Size variants profile obtained in SEC for Fc-fusion proteins using both non-volatile mobile phase and volatile mobile phase. Adapted from [90], with permission

and regulatory agencies ask for complementary methods, such as analytical ultra-centrifugation (AUC) or light scattering [81,86].

2.3.1 | Forced degradation studies

The modifications that proteins can undergo are considered as degradation products and can be classified either as product-related substances or as impurities. When the degradation product has properties not comparable to those of the desired product, it will be considered as an impurity that may eventually affect the activity, efficacy and safety of the product [93]. Many external factors can contribute to the formation of degradation products, e.g. pH, temperature, light, exposition to surface, storage time, salt concentration, protein concentration, formulation excipients or shaking, as well as intrinsic factors, such as free thiols in the Fc-fusion protein moieties [36,81,82,94]. Due to these potential modifications, the determination of the biopharmaceutical drug purity is difficult to achieve. However, purity and integrity confirmation of the product are required by regulatory agencies [85,93]. According to the ICH guideline Q5C, tests for purity should focus on degradation products, as some of them are known to negatively affect the final drug quality, safety, and efficacy [35,95]. This guideline also indicates that degradation product formation could be established during long-term, accelerated, and/or stress stability studies. Among these possibilities, forced degradation studies are often preferred.

Aggregation, fragmentation, deamidation, oxidation, cyclization, sialylation of oligosaccharide residues, isomerization, change in the free thiols, changes in glycosylation, and glycation are all degradation pathways that could be investigated in forced degradation studies [85,96]. However, among the impurities, aggregate formation is the most widespread source of concern in biopharmaceuticals development, due to the increased risk of adverse immunogenic reactions [36,82]. Once formed, aggregates represent a group of heterogeneous protein molecules assembled in a higher order structure. Various forms of aggregates exist, differing by size, morphology, protein structure, type of intermolecular bonding, and reversibility. [81] Protein molecules interact through weak non-covalent bonds, resulting in reversible aggregate assemblies [36,94]. Aggregates may also be formed by irreversible intermolecular disulfide bonds between unpaired free thiols. The formation of other irreversible covalent linkages occurs less frequently [94].

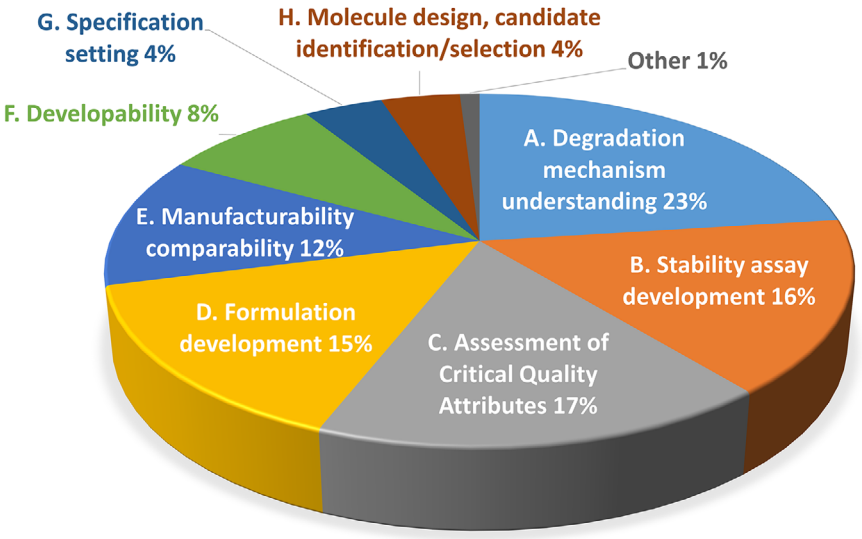

FIGURE 5 Reasons to perform forced degradation studies for the biopharmaceutical industry. Adapted from [96], with permission

During forced degradation studies, therapeutic proteins are exposed to stress conditions, e.g. thermal stress, shaking, freeze–thaw, chemical oxidation, photo stress, pH, and deamidation stress. These harsh conditions will lead to the formation of degradation products within a short period of time. There are many available options to perform forced degradation studies, such as different materials, conditions, number and duration of time points, as well as several analytical methods to evaluate the results of the applied stress. Halley et al. conducted an intercompany benchmarking survey and reported an overview of the common practices used to perform forced degradation studies [97]. Since Fc-fusion proteins possess a large structural heterogeneity and complexity, no specific stress and no generic conditions could be advised. Pre-screening studies are generally performed to find the best conditions and a compromise has to be found between too extreme conditions, not truly representative of real behaviors, and too gentle conditions, which could be unable to properly highlight degradation pathways and kinetics. Moreover, there is a lack of regulatory guidance on forced degradation procedures. Similarly, well-established acceptance criteria are missing, therefore limits are often defined by researchers based on previous experiments [83].

There are many reasons to perform forced degradation studies, as illustrated in Figure 5. At early stage of development, they can lead to a better discrimination and manufacturability evaluation of the drug candidate. They also have an essential role for the optimization of the formulation composition to ensure the best stability of the drug from storage to administration to the patient. Forced degradation studies allow the establishment of suitable stability indicating methods, i.e., methods able to detect specific degradations products. In addition, when performing forced degradation studies, the principle aim of the biopharmaceutical companies is to get valuable information about the degradation pathways (through the characterization of degradation products) and to highlight impurities which could also be defined as critical quality attributes (CQAs), as they can alter the product quality [96,97].

2.4 | Glycan analysis and sialic acids content

Glycosylation has an important influence on the immunogenicity and clinical efficacy of therapeutic proteins and is considered as a CQA for IgG and IgG-based therapeutics. Therefore, regulatory agencies demand glycoprotein manufacturers to determine the carbohydrate content (neutral sugars, amino sugars, and sialic acids), the oligosaccharide pattern (antennary profile), and the glycosylation sites on the polypeptide chain [98]. However, due to the absence of a direct genomic blueprint and the large intrinsic heterogeneity of glycosylation, a wide variety of orthogonal analytical techniques is required to fully elucidate the complex glycosylation profiles of therapeutic proteins.

To reduce the sample complexity, glycoprotein analysis is performed by using three main approaches corresponding to the size of the protein during analysis: intact and subunits protein level (top and middle-up, respectively), glycopeptides (bottom-up), and released glycans [99]. Subsequent analytical characterization is performed by using a wide variety of techniques, including, lectin microarrays, RPLC, HILIC, AEX, porous graphitic carbon (PGC), or CE, often hyphenated with MALDI-/ESI-MS, amperometric, or fluorescence detection (FD). Recent reviews have extensively described the use of these techniques for the analysis of Fc glycosylation of therapeutic mAbs and their biosimilars [100–105]. However, the glycan

profiles of Fc-fusion proteins are often more complex and extensive, with multiple N-glycosylation sites and putative additional *O*-glycans. Therefore, techniques that are applicable to mAbs are not always sufficient for the analysis of Fc-fusion proteins. Here, we will focus on reference techniques (generally used in method development) and specific analytical approaches that are of interest for the analysis of Fc-fusion proteins glycans profile.

HILIC analysis of fluorescently labeled released *N*-glycans is the most commonly used technique in glycan analysis for both quality control and method development purposes. The *N*-glycans are enzymatically removed from the glycoprotein using, e.g. peptide-*N*-glycosidase F (PNGase F), and fluorescently labeled via a reductive amination reaction with, e.g. 2-aminobenzamide (2-AB) [106]. Subsequently, the labeled glycans are separated using an HILIC column and identified based on a 2-AB labeled dextran ladder that provides the number of glucose units (GU) of each species [107,108]. However, drawbacks of the HILIC(2-AB) method include the long sample preparation procedure (~40 h) and co-elution of species in heterogeneous glycosylation patterns that can hamper both qualification and quantification [109,110]. An interesting approach to circumvent these problems is the use of RapiFluor-MS labeling in combination with HILIC-FD detection and MS identification. The use of RapiFluor-MS labeling significantly reduces the sample preparation time (~1 h) and improves sensitivity in both fluorescence and MS detection [111]. Moreover, the addition of MS detection provides a fully orthogonal identification method to FD and provides a more confident characterization and quantification in case of co-eluting species. [112–114] This is of particular interest when analyzing the more complex (bi-, tri-, tetra-antennary) N-glycan structures released from Fc-fusion proteins.

Together with the more complex N-glycan structures comes a higher sialic acid content that plays an important role in the immunogenicity and pharmacokinetics (PK) parameters of Fc-fusion proteins. Sialic acid profiling can be performed with the released and labelled glycans, using AEX chromatography that cleverly makes use of the negative charges introduced by, e.g. N-Acetyl neuraminic acid (Neu5Ac aka NANA) and non-human N-Glycolyl neuraminic acid (Neu5Gc aka NGNA) [115,116]. This method allows separating neutral glycans (non-sialylated) from mono-, di-, and tri-sialylated glycans [24]. Recently, Largy et al. reported an AEX-RPLC approach that uses the two orthogonal separation techniques to separate glycan species (that were co-eluting in HILIC mode) of etanercept based on the presence of sialic acid (Figure 6) [112]. It is worth mentioning that these techniques are not able to distinguish various types of sialic acids, such as Neu5Ac and Neu5Gc. Due to the immunogenic effects of the non-human Neu5Gc, a second complementary approach should be applied to characterize sialic acids [117,118]. This approach often includes the chemical release of sialic acid from the glycans and fluorescent labeling with DMB, prior RPLC chromatography with FD detection [119]. For many of the Fc-fusion proteins, such as etanercept and CTLA4-Fc, the sialic acid content was not only found on the N-glycans but also on O-glycans [24,40,41,53]. Indeed, in addition to N-glycans, Fc-fusion proteins often carry several O-glycosylation sites in the non-IgG domain. O-linked glycans require a different approach because they cannot be enzymatically cleaved using PNGase F. A common way to remove the O-glycans is via reductive β-elimination, after which, analysis can be performed with PGC-ESI-MS or MALDI-TOF [101,120,121]. This allows the challenging characterization of O-glycan structures, which in contrast to N-glycans, have highly diverse core structures [122]. However, O-glycans can be randomly attached to hydroxyl groups of amino acids, e.g. serine and threonine, thereby creating a high amount of putative O-glycosylation sites on the Fc-fusion protein. Therefore, obtaining site-specific information on the glycan attachment sites is important to accurately analyze the glycan profile.

A major limitation of the released glycan approach is the loss of site-specific information. Site-specific glycan patterns can provide important information on the safety and efficacy of Fc-fusion products that often contain multiple N- and O- glycosylation sites [24,25,40,41,53]. Recently, Higel et al. demonstrated that there are PK differences when the same glycan profile is located in the IgG-domain or in the fused partner domain of the Fc-fusion product [27]. Therefore, the site-specific information on the glycosylation pattern is of pivotal importance to further develop the clinical potential of therapeutic products and provide a better understanding of the CQAs.

The site-specific information is predominantly obtained via peptide mapping techniques, where the glycoprotein is enzymatically digested using proteases (e.g., trypsin) to generate peptides and glycopeptides of approximately 0.5-5 kDa. The obtained peptides are then analyzed using MALDI-MS or ESI-MS to obtain the glycan profile based on the accurate mass information [102,123]. To overcome the ion suppression effects of regular peptides and high-abundant glycopeptides, MS detection is often preceded by a chromatographic (e.g., RPLC and HILIC) or electrophoretic separation technique (e.g., CE) to ensure confident characterization of low abundant glycoforms [123–125]. Moreover, tandem MS analysis (e.g., electron-transfer dissociation) of the glycopeptides is used to identify the glycan site and determine the occupancy. This site-specific glycan occupancy information is considered as highly valuable information for Fc-fusion proteins that contain O-glycans, lacking a single consensus sequence as

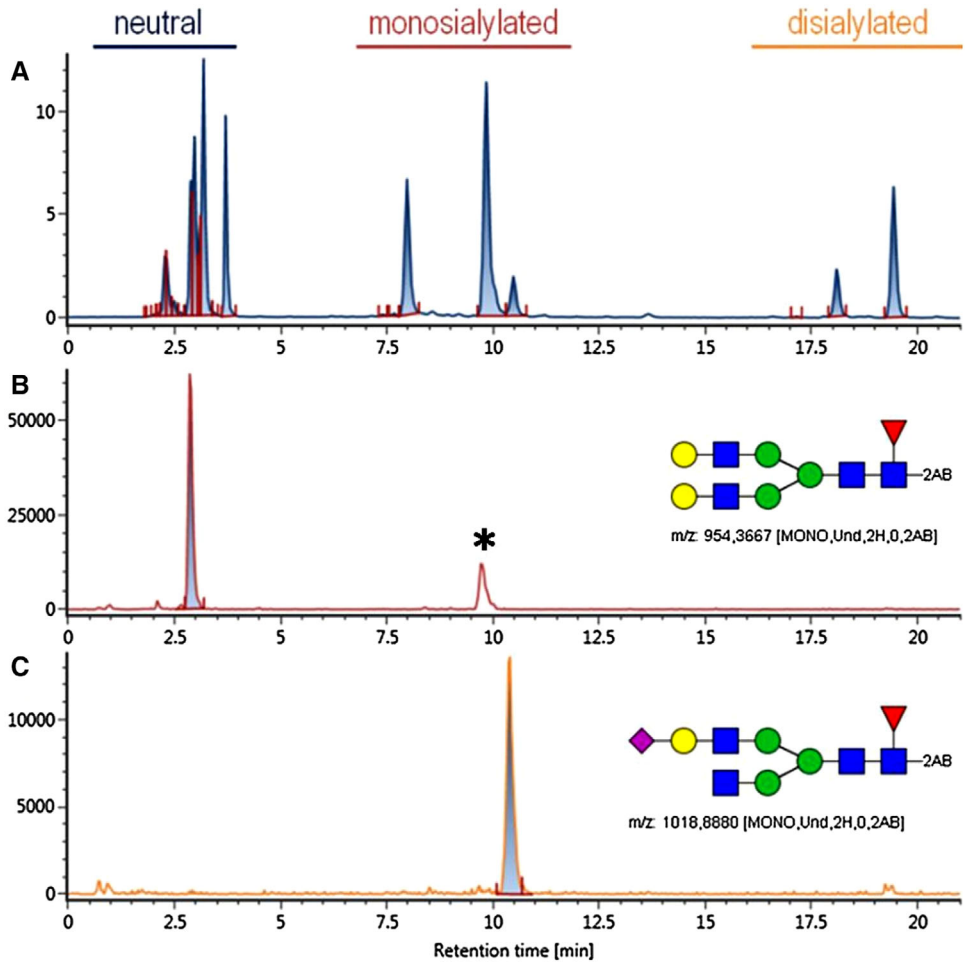

FIGURE 6 Fluorescence chromatogram of AEX-RP separated 2-AB labeled glycans of etanercept (A). The MS1 extractions of G2F (B) and G1FS1 (C) confirm the separation based on the sialylation level of the glycan species. The asterisk corresponds to in-source fragment of G1FS1 after loss of the sialic acid moiety. Adapted from [112], with permission

glycosylation site and that therefore could be distributed randomly across the protein.

Another interesting new concept for glycan analysis is the middle-up approach that deals with protein subunits (~25-100 kDa) obtained after enzymatic digestion (e.g. *IdeS*, Papain) and/or chemical reduction (e.g. DTT, TCEP) [66,126]. After the recent introduction of wide-pore HILIC amide stationary phases, HILIC-FD-MS analysis has shown to provide sufficient resolving power for qualitative glycosylation profiling of mAbs and Fc-fusion proteins [49,53]. A clear benefit of the middle-up strategy is that important site-specific glycan information can be obtained with limited sample preparation procedure, when compared to the bottom-up approach [66,67]. Moreover, a novel 3D-LC/MS approach was recently introduced to perform middle-up level glycan analysis of mAbs in a fully automated workflow [50]. Therefore, middle-up analysis provides an interesting alternative for routine analysis or rapid batch-to-batch comparisons of glycosylation profiles of therapeutic proteins.

To conclude, a plethora of different techniques are available for the analysis of both N- and O-glycans of Fc-fusion proteins. Special attention should be paid to the multiple glycosylation sites, the site-specific glycosylation profile and the presence of both N- and O-glycans. Therefore, it is important to use a combination of orthogonal techniques that provide complementary information on the complex glycosylation patterns of Fc-fusion proteins. The combined results can be used to fulfill the elaborate demands of the regulatory agencies.

3 | CASE STUDIES RELATED TO THE ANALYTICAL CHARACTERIZATION OF FC-FUSION PROTEINS

The currently marketed Fc-fusion proteins (Table 1) can be classified in four main categories based on the functional diversity related to the ligand-binding domain that can be derived from a receptor ECD, a cytokine trap, a

peptide, or an enzyme (Figure 1). ECD-Fc fusion proteins (Section 3.1) are the most represented category. Their ligand-binding domain consists of the ECD of a natural receptor acting as receptor antagonist with a single-ligand specificity. This category includes etanercept, alefacept, abatacept, belatacept, and luspatercept. Cytokines traps (Section 3.2) are receptor antagonists consisting of genetically engineered binding constructs that have multi-ligand specificity. Rilonacept, aflibercept, ziv-aflibercept, and conbercept belong to this category. Peptide-Fc fusion proteins or peptibodies (Section 3.3) are represented by romiplostim and dulaglutide. The ligand-binding domain of this category consists of functionally active peptides that act as receptor agonists and have single-ligand specificity. Finally, efmoroctocog alfa, eftrenonacog alfa, and asfotase alfa belong to the enzyme-Fc fusion proteins (Section 3.4) category characterized by a ligand-binding domain consisting of enzyme substitutes with single-ligand specificity.

This section aims to report the main analytical strategies applied for the characterization of the selected Fc-fusion proteins based on the characteristics of their ligand binding domains.

3.1 | ECD-Fc

3.1.1 | TNFR-Fc

Etanercept is a ~130 kDa soluble Fc-fusion protein consisting of the ECD of the human p75 TNFR coupled to a human Fc-IgG1 domain. The dimeric Fc-fusion protein holds a canonical N-linked glycosylation site in the Fc-domain and two N-linked glycosylation sites in the TNFR-domain. In addition, thirteen O-linked glycosylation sites have been identified in the linker domain [24]. Together, the N- and O-linked glycans (~30 kDa) create a highly heterogeneous glycosylation profile, which is considered as an important CQA of etanercept. Further structural complexity is added by the thirteen intra-chain disulfide bonds and the three inter-chain disulfide bonds holding the monomers together [127]. Therefore, the comprehensive characterization of etanercept presents a significant analytical challenge and requires a wide variety of analytical techniques.

To characterize the highly complex N- and O-linked glycan profile of etanercept, Houel et al. used an interesting approach combining specific sample preparation procedures with the use of LC–FD and LC–MS/MS analytical platforms [24]. A released glycan approach was used in combination with an exoglycosidase array digestion to identify the N- and O-glycan patterns on a HILIC-FD platform. For the N-glycans, PNGase F and 2-AB labeling were applied before analysis, while β-elimination was used for releasing the O-glycans. By combining these released glycan approaches, important information on the N- and O-linked glycans present on etanercept was discovered, even though site-specific information was not obtained. For this reason, etanercept was enzymatically digested with *IdeS* to separate the TNFR- and Fc-domains prior to site-specific N-glycosylation analysis using the previously described released glycan approach. Site-specific information on the O-glycans was obtained from a tryptic digest of etanercept after treatment with PNGase F and sialidase to remove both N-glycans and terminal sialic acids. The remaining O-glycopeptides, consisting only of a core-1 O-glycan (i.e. Galβ1-3GalNAc) and peptide backbone, were used to identify the O-glycosylation sites by RPLC-MS^E with electron-transfer dissociation (ETD).

Montacir et al. used a similar peptide mapping approach for the site-specific analysis of both N- and O-linked glycans on etanercept and a biosimilar product (Altebrel) [128]. After proteolytic digestion, the non-glycosylated peptides were separated from glycopeptides to reduce the negative effects on the ionization efficiency and eliminate the need for terminal sialic acid cleavage. This was achieved by a HILIC-based purification technique that selectively extracts the N- and O-glycopeptides prior to RPLC-MS^E analysis. The MS-based identification of both the N- and O- glycan profile was further improved by the use of collision-induced dissociation (CID) to obtain product–ion spectra of all glycopeptides at high-energy. The obtained glycosylation sites were comparable to findings from Srikanth et al. that labeled the N- and O-glycopeptides with ^{18}O-water during digestion and analyzed the glycopeptides using a RPLC–MS/MS with multiple-reaction monitoring (MRM) [129]. With the latter approach, a targeted site-specific quantitative analysis of the N- and O-glycopeptides of etanercept was obtained. It is worth mentioning that this targeted approach is limited for glycoproteins from which the glycan structures are not known a priori.

Wohlschlager et al. used a native MS approach in combination with a judicious enzymatic digestion procedure to provide information on the true glycoform heterogeneity at the intact and subunit protein level [130]. However, accurate annotation of the glycan pattern after the enzymatic digestions remained limited due to the inability of the MS to separate glycoforms having small mass differences. D'Atri et al. showed that the addition of a HILIC separation step prior to MS detection can greatly increase the resolution when analyzing the glycoprotein at subunit level [53]. By using a combination of enzymatic digestions (e.g. glycosidase, protease, sialidase) prior to HILIC-QTOF-MS, subunit-specific N- and O-glycan patterns were obtained for etanercept and additional PTMs were identified. Therefore, this approach provides a much simpler strategy compared to peptide mapping and

a viable alternative to the native MS approach that was performed with Orbitrap MS technology.

Another important structural aspect of etanercept is the level of incorrect disulfide bridging that has been related to a reduced potency in biological assays [131]. Characterization of the disulfide bonds can be performed using a peptide mapping approach in which deglycosylated and desialylated etanercept is digested under non-reducing conditions. This creates disulfide linked peptides that can be detected using RPLC-MS/MS and provide valuable information on the 13 intra-chain and 3 inter-chain disulfide bonds. A similar peptide mapping approach can be used to determine other PTMs that result in sequence variants e.g. oxidation and deamidation [127].

3.1.2 | CTLA4-Fc

In 2005, abatacept (Orencia®) was the first CTLA4-Fc fusion protein approved by the FDA for the treatment of patients with rheumatoid arthritis [132], while belatacept (Nujolix®, which only differs by two amino acids form abatacept) was approved in 2011 as prophylaxis for organ rejection in adult patients receiving a kidney transplant [133]. The CTLA4-Fc fusion proteins are genetically engineered fusion proteins composed of the extracellular domain of human CTLA4 linked to the modified Fc (i.e., hinge, CH_2, and CH_3 domains) portion of human IgG1. They have a molecular weight of around 80 kDa. Their production in Chinese hamster ovary (CHO) cells results in a macro- and micro-heterogeneous glycosylation pattern, with multiple sites of N- and O-linked glycosylation [134,135]. To date, three N-glycosylation sites (two in the CTLA4 domain and one in the Fc region), plus four O-glycosylation sites in the hinge region (see Supporting Information) have been reported [135]. Therefore, CTLA4-Fc fusion proteins are highly glycosylated and complex species, requiring the use of a wide range of analytical techniques applied at multiple levels of analysis to allow a full and comprehensive characterization.

Bongers et al. described a LC–MS/MS peptide mapping approach for the characterization of glycosylation profile of a CTLA4-Fc fusion protein without removing the attached glycans [23]. In this study, the two N-glycosylation sites on each CTLA4 domain (Asn-76 and Asn-108), the O-glycosylation site in the hinge region (Ser-129), and the single N-linked site at Asn-207 on the Fc domain were characterized. For this purpose, the authors reported the use of ESI in-source CID, for the location of both N- and O-glycosylation sites by selected-ion-monitoring of carbohydrate oxonium fragment ions. Many glycoforms were then identified at each site via MS/MS, and their sites-occupancy percentages were measured. Based on this data, the authors observed that Asn-108 site contains N-glycans with overall higher levels of terminal galactose and sialic acid versus the Asn-76 site, while the latter contains multiple structures with terminal GlcNAc residues and higher levels of terminal GlcNAc. The peptide mapping approach enabled to differentiate peculiar glycan populations at each site of the CTLA4-Fc fusion protein, which is typically challenging with the conventional released N-glycan methods such as 2-AB labeling HPLC.

To circumvent tedious sample preparation steps that are typically required with the standard peptide mapping approach, Lynaugh et al. developed a rapid and simple procedure combining *IdeS* digestion with LC/MS for Fc glycosylation analysis of abatacept at the middle-up level [136]. First, the authors mentioned that the LC/MS analysis of intact abatacept provided a spectrum that cannot be deconvoluted, probably due to the high heterogeneity of glycosylation. Then, the middle-up approach was applied and allowed to determine the structure and relative composition of each N-linked Fc glycan, where the identity of sialylated N-glycans was confirmed by treating the sample with neuraminidase. In addition, the analysis of an *IdeS* and PNGase F treated sample allowed to identify the aglycosylated Fc fragment, as well as three other peaks with 324 Da mass additions to the aglycosylated Fc fragments. Based on these results, the authors demonstrated the presence of a maltose glycation that resulted from the storage of reconstituted abatacept powder formulated with maltose. Interestingly, the middle-up approach enabled to confirm the detection of uncommon α-galactosylation present in the Fc region of abatacept, which demonstrates its interest for characterization of Fc proteins with a mutated Fc hinge region.

Recently, Zhu et al. highlighted the advantages of combining the multiple levels of CTLA4-Fc fusion protein analysis, i.e. intact, reduced, middle-up, and peptide level, enabling the full characterization of glycosylation modifications to have a comprehensive level of knowledge about the biosimilar candidates [40]. For this purpose, the authors showed the versatility of the LC coupled to fluorescence detection and MS. LC/MS analysis at intact level was performed, enabling to observe that the biosimilar and innovator had the same retention time, even though detailed information about glycosylation heterogeneity was challenging. Therefore, subunits analysis was performed after DTT reduction to decrease the complexity. However, the authors were still faced with the high complexity of the CTLA4-Fc fusion proteins due to the presence of multiple N- and O-glycosylation sites. The application of (i) PNGase-F to remove the N-glycans, and the (ii) combination PNGase-F and neuraminidase to remove both N-glycans and the sialic acids enabled

to further characterize the O-glycans at subunit level. Based on these data, the authors confirmed that biosimilar and control have complex profiles, both containing three N- and four O-glycosylation sites on each chain. The three main N-glycan forms were G0F, G1F, and G2F, while the O-glycan forms were composed of one N-acetylgalactosamine and one galactose, with one or two sialic acids. In addition, LC-MS^E peptide mapping approach enabled to site-specifically locate the glycans, consisting of two N-linked glycosylation sites on CTLA4 domain (Asn-76 and Asn-108) and one on the Fc domain (Asn-207), while four O-linked glycosylation sites were located in the hinge region (Ser-129, Ser-136, Ser-139, and Ser-148). For the first time, the authors reported the identification of four O-glycosylation sites in the CTLA4-Fc fusion protein using the ETD fragmentation technique. Further characterization of the glycosylation profile was performed using a HILIC-fluorescence method for accurate quantification of the released N-glycans. Small differences in the relative rates of G2FS1 and G2FS2 between the innovator and the biosimilar CTLA4-Fc fusion proteins were observed and isomers of glycans such as G1a and G1b, and G1Fa and G1Fb were resolved. Overall, the versatility of the LC/MS method allowed identifying the similarity of a candidate biosimilar CTLA4-Fc fusion protein including (i) the structures of N-glycans, (ii) the heterogeneity of N-glycosylation sites, and (iii) the heterogeneity of O-glycans. For biosimilar development programs, this type of information can strongly influence decisions about the type and amount of animal and clinical data needed.

3.2 | Cytokines traps

Blocking the activity of cytokines (e.g. TNF-a, IL-1, IL-4, IL-6) has a great clinical potential in a variety of disease pathologies. However, the use of a single extracellular domain (i.e. as used in etanercept) to neutralize a cytokine is often not sufficient, due to the complex multicomponent receptor systems involved [137]. Therefore, in cytokine-traps, ligand binding domains of multiple receptors are combined to create a high-affinity monovalent binding site to trap a cytokine-ligand. This technique was first applied in rilonacept (~251 kDa), a cytokine-trap that couple the C-terminal end of the IL1 receptor accessory protein (IL-1RAcP) binding domain to the N-terminal end of the IL-1R 1 extracellular binding domain [138,139]. Coupling the resulting hybrid IL-1 binding domains to each N-terminal end of the human IgG1-Fc domain creates a highly potent IL-1R antagonistic drug that is slowly cleared due to the PK characteristics of the IgG1-Fc domain [140]. A similar approach was used for both (ziv-) aflibercept (~115 kDa) and conbercept (~143 kDa) that consist of a hybrid binding domain from vascular endothelial growth factor (VEGF) receptors 1 and 2 fused to a human IgG1-Fc domain [138,141]. In the smaller aflibercept, the second domain of VEGFR1 and third domain of VEGFR2 are combined in a single chain and connected to the Fc-domain. With two N-glycosylation sites per receptor-domain and a single conserved N-glycosylation site on the Fc-domain, aflibercept has a complex glycan profile with ten oligosaccharide structures attached to the protein. Significant for conbercept is the addition of VEGFR2 domain 4, that is not essential for the ligand binding, but stabilizes the receptor structure upon binding of VEGF. The additional domain adds two more N-glycosylation sites to each monomer and create a highly complex glycan profile with a total of 14 potential N-glycans attached to the protein. Both (ziv-)aflibercept and conbercept have anti-angiogenetic properties via the neutralization of VEGF-A, VGEF-B, and the placental growth factor (PlGF) and are used for treatment of several retinal diseases [141].

The currently approved cytokine-traps are all homodimeric proteins that consist of fully human amino acid sequences that represent the hybrid binding domain and the coupled IgG1-Fc domain. Furthermore, all cytokine-traps are glycoproteins which are expressed in Chinese Hamster Ovary (CHO) mammalian cell lines to ensure the correct glycosylation profiles on both the ligand-binding domain and the IgG1-Fc domain [142–144]. The combination of the complex glycosylation profiles and the multicomponent binding domain makes the structural characterization challenging and require an array of analytical techniques.

This was well illustrated by Wu et al. who performed a structural characterization of conbercept using a variety of analytical techniques. It was demonstrated that ~30% of the total MW of conbercept is accounted for by the glycan structures, compared to ~15% in aflibercept and 25% in rilonacept [141,142,145]. By using conventional bottom-up approaches, the amino acid sequence and correct disulfide bridging of conbercept were confirmed and seven N-glycosylation sites were identified on each protein monomer. Subsequent glycan profiling was performed using a combination of glycopeptide analysis by RPLC-MS and released glycan analysis using AEX with FD, to obtain both qualitative and quantitative information on the oligosaccharide structures attached to the protein. In addition, icIEF was used to determine the isoelectric point (*pI*) of conbercept and showed a strong influence of glycosylation on the *pI* of the protein and a large amount of charge isoforms. To elucidate the effect of glycosylation on the charge isomers of conbercept, Li et al. presented an interesting approach combining 2DE with a site-specific and quantitative LC-MS/MS glycopeptide analysis strategy (Figure 7) [146]. High-resolution 2-DE was used to separate

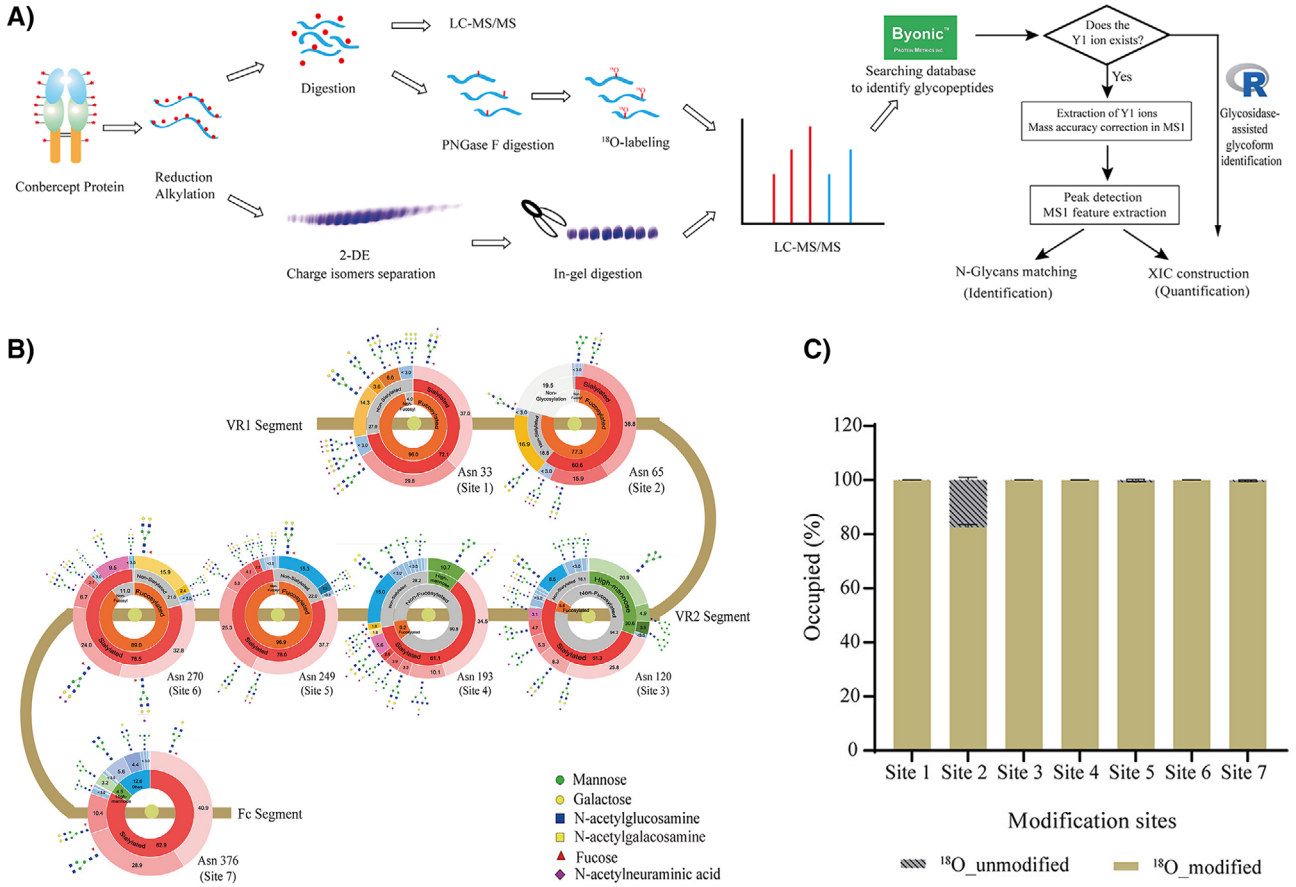

FIGURE 7 Effect of glycosylation on the charge isomers of conbercept. (A) Analysis workflow for the 2-DE, digestion and ^{18}O-labeling processes; (B) Distribution of site-specific N-glycans at seven sites; (C) Results of occupancy ratios of N-glycans at each site form nine batches of conbercept. Adapted from [146], with permission

the charge isoforms based on their differences in *pI* and MW using a gel strip with an immobilized pH gradient. Then, the obtained gel spots, representing the different charge variants, were subjected to in-gel tryptic digestion to create the glycopeptides used in the subsequent LC–MS/MS analysis. The site-specific and quantitative information on the N-glycan heterogeneity was obtained with a compelling Y1 ion-guided MS strategy. MS/MS fragmentation creates Y1-ions (i.e. peptide backbone + core GlcNAc) which are specific for each N-glycosylation site, due to the attached peptide sequence. By extracting the MS2 profile of these Y1-ions, the MS1 retention time range of the N-glycopeptides that is specific for a single N-glycosylation site, can be determined. In this range, qualitative glycan profiling can be performed based on the diagnostic fragment information of N-glycopeptides bearing different glycoforms. Furthermore, the relative quantitation of the site-specific glycan profile was obtained by comparison of the intensities in deconvoluted spectra of different N-glycoforms after normalization using the most abundant species. The analysis of the 2-DE gel spots demonstrated that glycan species with a higher sialic acid content were more abundant on the charge isomers located toward the acidic terminal. In addition, the quantitative approach showed that one of the seven N-glycosylation sites had 20% lower glycan occupancy compared to the other sites and confirmed the results of the ^{18}O-labeled site occupancy experiments. At last, the approach was applied to nine different batches of conbercept and demonstrated good consistency in analytical performance. Therefore, the Y1-ion guided MS1 site-specific glycopeptide quantitation strategy could be of particular interest for routine batch-to-batch analysis and biosimilar comparison of other fusion proteins that are heavily glycosylated.

3.3 | Peptide-Fc (peptibodies)

Peptibodies are chimeric proteins consisting of a biologically active peptide conjugated to an Fc-domain of IgG [147]. Conjugation to the Fc-domain provides to the active peptide an extended half-life via FcRn-mediated recycling and reduced renal clearance [2]. Moreover, by conjugating multiple peptides to a single Fc-domain the avidity is

increased and a higher clinical efficacy can be obtained. Currently, two peptibodies are approved by the EMA and FDA: romiplostim (Nplate®) and dulaglutide (Trulicity®) [147]. Romiplostim (~60 kDa) is a dimeric peptibody comprised an aglycosylated IgG1 Fc-domain (*E. coli* production) fused with two polypeptide mimetic sequences of thrombopoietin (TPO) attached to each Fc chain [148]. Romiplostim is structurally different from all the other Fc-fusion products due to the binding of the biologically active peptide to the C-terminal end of the Fc-domain. Dulaglutide (~63 kDa) is comprised of two glucagon-like peptide-1 (GLP-1) receptor agonists linked to the N-terminal ends of the IgG4 Fc-domain. By using the Fc-domain of IgG4 the immune-mediated ADCC and CDC effector functions are reduced, and administration is only required once a week for treatment of type 2 diabetes mellitus [148].

Compared to most ECD-Fc fusion proteins, peptibodies are significantly less complex to analyze due to their smaller size (~60 kDa) in combination with little to no N-linked glycans (*E. coli* production). Therefore, the analytical characterization of peptibodies can be performed using a conventional bottom-up approach with LC–MS/MS [149]. Another interesting approach is the middle-up analysis of peptibodies. Chemical reduction of the disulfide bonds between the single Fc-chains creates subunits of around 30 kDa that can be easily analyzed using RPLC- and HILIC-MS for the most common PTMs [67]. Further size reduction of the subunits by enzymatic digestion, using *IdeS*, has little added value for romiplostim, since the biological active peptide is coupled to the C-terminal end of the Fc-domain. Moreover, the application of *IdeS* to dulaglutide is hampered by the so-called LALA double mutation of the hinge region (Leu234Ala together with Leu235Ala to abrogate ADCC) and therefore requires the use of the more specific *IdeZ* enzyme (unpublished data) [150]. This would result in subunits of ~6 and ~25 kDa for the GLP-1 analog and Fc/2, respectively. In addition, a complementary top-down approach is recommended to analyze the product as a whole and exclude potential artefacts introduced from the digestion process [151]. This can be of particular interest when studying the proteolytic degradation sites and PK properties of peptibodies in vivo [149].

3.4 | Enzyme-Fc

Factor VIII (FVIII) and Factor IX (FIX) are blood-clotting enzymes that play a crucial role in the coagulation cascade. A deficiency in coagulation enzyme activity results in bleeding disorders known as Hemophilia A (deficiency of FVIIII) and Hemophilia B (deficiency of FIX). As recommended standard of care, Hemophilia A/B is treated by intravenous injections of either recombinant or plasma-derived Factor proteins (rFVIII/IX and pdFVIII/IX, respectively). However, this prophylactic therapy requires repeated intravenous infusions, due to the relative short half-life of FVIII and FIX [152]. ref Fusion of an IgG-Fc domain to the rFVIII (rFVIII-Fc, efmoroctocog α) and rFIX (rFIX-Fc, eftrenonacog α) has shown to reduce the clearance of these factors, while retaining the correct biological activity, thus obtaining a second-generation therapy with significantly improved clinical value that enables patients to reduce the number of drug administration [153–156].

A structural peculiarity of clotting Factor-Fc proteins is their monomeric configuration, meaning that a single effector molecule is fused to the dimeric Fc region, in contrast to the traditional dimeric Fc-fusion proteins bearing two effector molecules, one on each Fc chain. This monomeric configuration was found to unexpectedly improve the pharmacokinetic (PK) and pharmacodynamic (PD) profiles of rFVIII/IX [14,152,157,158].

From an analytical point of view, the rFIX-Fc effector molecule (~50 kDa) is highly heterogeneous and undergoes extensive posttranslational modifications. Besides two *N*-linked and six *O*-linked glycosylation sites, it shows characteristic PTMs, such as several modifications of glutamate (Glu) to γ-carboxyglutamate (Gla), β-hydroxylation of an aspartic acid residue, sulfation of a tyrosine residue, and phosphorylation of two serine residues (for a detailed list of the modified residues, the readers can refer to [154,159] and Supporting Information). As reported by Peters et al., several analytical approaches might be required for the complete characterization of all these PTMs, while peptide mapping and released glycan analysis are generally the most effective approaches [157]. It should be noted that Gla residues play a pivotal role for the rFIX function within the blood clotting cascade and therefore the analysis of the γ-carboxylation content of the rFIX-Fc is of utmost importance. A 12 Gla/rFIX ratio is generally expected. The Gla content can be confirmed by MS after enzymatic digestion and subsequent peptide mapping. Peters et al. also performed Gla residue evaluation by total amino acid analysis after base hydrolysis of rFIX-Fc and comparison of the results with those for plasma-derived FIX (pdFIX) [157]. Amari et al. developed several analytical methods to monitor Gla content, including amino acid analysis (total Gla content), peptide mapping by LC–MS/MS (Gla occupation), and FIX coagulation activity by activated partial thromboplastin time (aPTT) and analytical AEX (isoform separation) [160].

The rFVIII-Fc effector molecule has an MW of ~170 kDa (organized as a 90 kDa HC and an 80 kDa LC), making it the largest biologic ligand linked to both a monomeric or dimeric Fc-fusion configuration to date [161,162]. Despite its size, the rFVIII and Fc components of rFVIII-Fc were

reported to be structurally indistinguishable from their isolated constituents. Five orthogonal methods were used to characterize the structure of rFVIII-Fc compared to rFVIII, namely hydrogen–deuterium exchange MS (HDX-MS), X-ray crystallography, small-angle X-ray scattering (SAXS), electron microscopy (EM), and surface plasmon resonance (SPR) [163]. Similar to the rFIX-Fc effector molecule, rFVIII undergoes extensive PTMs, with four potential N-linked glycosylation sites and the sulfation of six tyrosine residues (for a detailed list of the modified residues, the readers can refer to [153,159] and Supporting Information. Note that rFVIII has a reduced size in respect to the full-length FVIII that shows 20 additional N-linked glycosylation sites). As reported for rFIX-Fc, the rFVIII tyrosine sulfation can be determined by amino acid analysis (base hydrolysis) relative to pdFVIII [157].

Besides the characterization of the PTMs, and given the structural complexity of the clotting Factors-Fc proteins, process validation studies are required to evaluate identity, purity, activity, and safety. Furthermore, the manufacturing process has to ensure consistent product quality and high purity of the clotting Factors-Fc proteins [164–166].

4 | BIOSIMILARS OF FC-FUSION PROTEINS

Biosimilars are biologic drugs designed to be highly similar to the approved reference innovator biologic therapeutic agent. The European Medicines Agency (EMA) approved the first biosimilar mAb in 2013 [167]. Currently, near 30 mAb biosimilars have been approved in the European Union (EU) as well as three biosimilars of etanercept [105,168]. These successful candidates have undergone rigorous analytical, nonclinical, and clinical evaluations to demonstrate similarity to the approved biologic [95,98,169]. It should be noted that there are some biological products that claim biosimilarity but have not undergone the rigorous characterization and testing as described in World Health Organization (WHO) guidelines. These products are available outside the European Union and the United States of America and may have unintentional structural or chemical differences, and therefore are not biosimilars; they are termed intended copies (ICs), biomimics, or non-comparable biotherapeutic products. With the etanercept patent expiration in the EU in 2015, several copies are undergoing clinical trials, with the goal of providing cheaper alternatives to the reference product. [170] Due to the complex structure of etanercept, analytical challenges are limiting the development and approval of biosimilars. Many biosimilar candidates of etanercept are investigated in clinical trials worldwide, but so far, only three reached the market in the EU and the United States (Table 3).

TABLE 3 Etanercept biosimilars approved in the European Union and the United States

Biosimilar	Company	EMA approval	FDA approval
Benepali® (SB4)	Biogen, Samsung Bioepis	2016	2019 Eticovo® (etanercept-ykro)
Erelzi® (GP2015)	Sandoz (Novartis)	2017	2016 (etanercept-szzs)
Nepexto®	Mylan, Lupin	2020	n.a.

SB4 was developed as a biosimilar to Enbrel® and was approved as Benepali® (Biogen), the first biosimilar of etanercept licensed in the EU in 2016. SB4 was also approved as Eticovo® (etanercept-ykro, Samsung Biopepis) in the USA in 2019. The quality assessment of SB4 was performed in accordance with the ICH comparability guideline and the biosimilar guidelines of both the EMA and Food and Drug Administration (FDA). Extensive structural, physicochemical, and biological testing was performed with state-of-the-art technologies during a side-by-side comparison of the products [171]. Noteworthy, similarity of critical quality attributes (CQAs), including N- and O-glycans, was evaluated based on tolerance intervals established from quality data obtained from more than 60 lots of EU-sourced and US-sourced etanercept [53,105,127,172].

GP2015 (Sandoz/Novartis) was approved as Erelzi® in 2016 in the USA (etanercept-szzs) and in 2017 in the EU. In *in vitro* tests, GP2015 and Enbrel® had comparable binding affinities to TNF-α, C1q and a complete panel of Fc-Receptors. Comprehensive functional characterization testing confirmed the comparability of GP2015 with Enbrel® in terms of its ability to bind to and neutralize TNF-α, which reflects the primary mechanism of action of etanercept. Non-clinical data confirmed that the proposed biosimilar to Enbrel®, GP2015, is comparable with regards to its pharmacokinetic properties and pharmacodynamics activity, and efficacy as well as safety/toxicity. [173] In addition, clinical data showed a high level of similarity between the two products in accordance with regulatory requirements. The totality of the evidence from all analyses and performed trials was used to justify the use of the biosimilar in all indications for which the reference medicine is approved [174].

Intended copies (IC) of etanercept have also been approved in other countries such as China, Colombia, India, Iran and Mexico [128,175,176]. For example, batches of seven IC products of etanercept (Enbrel®) were subjected to a subset of test methods used in the routine release

and heightened characterization of Enbrel®, to determine key attributes of identity, quality, purity, strength, and activity. While several quality attributes of the tested IC lots met the release specifications for Enbrel®, none of them falls within these limits across all methods performed, and there were no IC lots that satisfied the criteria typically applied by the innovator to support comparability with Enbrel® [175].

5 | FUTURE PERSPECTIVES AND CONCLUSION

Since the approval of the first Fc-fusion protein (Etanercept) by the FDA in the 1990s, a wide variety of new therapeutics have been commercialized worldwide and Fc-fusion proteins can now be considered as one of the most successful classes of IgG-based products. As reported in this review paper, there is a very high structural diversity within the molecules belonging to this class, and despite the fact that the Fc part is common to all these products, the second part of the Fc-fusion protein (i.e. biological ligand) can be highly diverse in terms of size, charge, and hydrophobicity (i.e. it can be either extracellular domains of natural receptors, functionally active peptides, genetically engineered binding constructs acting as cytokine traps or even recombinant enzymes). This diversity explains why specific chromatographic, electrophoretic, and MS methods must be developed for Fc-fusion proteins. Besides this, the glycosylation profile of Fc-fusion proteins is highly complex involving the presence of multiple N- and O-glycans sites and numerous sialic acids, requiring highly innovative and complementary analytical methods.

To deal with the extreme complexity of Fc-fusion proteins, there will be a need to develop methods that are more powerful in the future. For example, multidimensional LC or ion mobility spectrometry–mass spectrometry, which have been poorly described until now for the analytical characterization of Fc-fusion proteins, can be useful. In addition, due to the presence of numerous sialic acids in their structures, it would be relevant to evaluate the interest for bioinert chromatographic systems and bioinert columns, to limit adsorption for charge variants analysis and glycan analysis. In general, AEX offers poor performance with Fc-fusion proteins, due to the very high number of charge variants and sialic acids. To further improve the amount of information that can be obtained from such analysis, MS compatible buffers must be developed in AEX. Finally yet importantly, we can also imagine that the production of new specific enzymes developed for this successful class of biopharmaceuticals could be helpful for their characterization at the sub-units level.

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CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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