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Review

Intact protein analysis in the biopharmaceutical field

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ABSTRACT

In recent years, a growing number of biopharmaceutical proteins have been produced and are already available, or will be soon available, in the market. These molecules are more complex to analyze than conventional low molecular weight drugs, and thus need powerful analytical approaches for the entire development and delivery process. This review summarizes the analytical techniques available for intact protein determination and the main development steps in which they are applicable. A strong emphasis has been put on separation techniques, liquid chromatography and electrophoretic techniques, but mass spectrometry and spectroscopic approaches are also mentioned. Overall, we highlight how several analytical strategies are necessary to obtain global information.

Contents

Abbreviations: AC, affinity chromatography; ACE, affinity capillary electrophoresis; ACN, acetonitrile; BGE, background electrolyte; CD, circular dichroism; CE, capillary electrophoresis; CEC, capillary electrochromatography; CGE, capillary gel electrophoresis; CIEF, capillary isoelectric focusing; CZE, capillary zone electrophoresis; DLS, dynamic light scattering; EIC, extracted ion chromatogramm; EOF, electroosmotic flow; ESI, electrospray ionization; FA, formic acid; FT-ICR, Fourier-transform ion cyclotron resonance; HILIC, hydrophilic interaction liquid chromatography; IEC, ion-exchange chromatography; IR, infrared spectroscopy; mAb, monoclonal antibody; MALDI, matrix-assisted laser desorption; MEEKC, micro-emulsion electrokinetic chromatography; MEKC, micellar electrokinetic chromatography; MS, mass spectrometry; NMR, nuclear magnetic resonance; PAGE, polyacrylamide gel electrophoresis; PEEK, polyether ether ketone; PLOT, porous layer open tubular; RPLC, reversed-phase liquid chromatography; SDS, sodium dodecyl sulfate; SEC, size-exclusion chromatography; SIM, single ion monitoring; TFA, trifluoroacetic acid; TOF, time-of-flight; UCA, ultrasound contrast agent; UHPLC, ultra-high pressure liquid chromatography.

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1. Introduction

During the last decade, the biotechnology sector has taken particular interest in pharmaceuticals. Insulin was the first product available from this area in 1982 [1,2], and the number of drugs originating from biotechnology is expected to reach 50% of all new chemical entities in a near future [3]. The development of these biomolecules is primarily related to the huge improvements in recombinant DNA technology. Indeed, only a small number of therapeutic proteins are purified from a native source, such as pancreatic enzymes from hog and pig pancreas [4]. In 2008, Leader et al. proposed a complete classification of all therapeutic proteins in current use, based on their pharmacological action [5]. Four groups were distinguished: therapeutic proteins with enzymatic or regulatory activity (e.g., insulin, growth hormone, and erythropoietin), those with special targeting activity (e.g., etanercept and abciximab), protein vaccines (e.g., hepatitis B surface antigen), and protein diagnostics (e.g., glucagon and growth hormone releasing hormone). The authors also called attention to the important potential of these proteins, considering that thousands of proteins are known to be produced by living organisms.

In terms of production, formulation, and quality control, therapeutic proteins pose many challenges compared to low molecular weight molecules because of their inherent complexity [6]. For instance, manufacturing of biopharmaceuticals requires a greater number of batches (>250 vs. <10), a larger number of product quality tests (>2000 vs. <100), and larger process data entries (>60,000 vs. <4000) [7]. The proteins' complexity is related to their numerous molecular weights, possible conformations, solubilities, stabilities, *in vivo* lifetimes, post-translational modifications, and microheterogeneity [8]. Concerning their heterogeneity, protein modifications could occur during production, extraction, purification, formulation, and storage. Small differences in manufacturing processes can affect the efficacy and safety of recombinant proteins [9]. Some heterogeneity is natural and has no consequences, but some variants could have adverse biological and clinical effects [10]. For example, variations in glycosylation influence the biological effect of erythropoietin [11]. Taking into account the number of co- and post-translational modifications it takes to "fine-tune" the activity of proteins (e.g., enzymatic cleavages, attachment of lipids, or glycans), it is consequently also complicated to produce a generic version of a biological drug [12]. Indeed, proteins require elaborate and sophisticated manufacturing processes, and their properties are highly dependent on the process employed. Legislation introduced in the U.S. Senate in March of 2009 describes a new regulatory pathway that would require manufacturers to demonstrate the following: (i) that generic versions of biological drugs are both very similar in molecular structure to the original one and share the same mechanism of action and (ii) that there are no significant clinical differences between the two products [13]. In this context, two distinct categories were defined: (i) "biosimilars", which include generic drugs that are merely similar to the brand-name drug and (ii) "biogenerics", which include generic drugs that are essentially identical to the brand-name drug, and thus can be substituted for the brand-name drug [13–16].

The specific characteristics of proteins compared to small molecules create the need for additional analytical methodologies. A variety of techniques including reversed-phase liquid chromatography (RPLC), size-exclusion chromatography (SEC), native gel electrophoresis and other electrophoretic techniques, mass spectrometry (MS), and UV and fluorescence spectroscopy have been used to study proteins in research, development, production, and quality control [3]. Analytical chemistry plays an important role in supporting these activities by helping to understand the impact that changes in manufacturing processes and scale have on the quality and consistency of the drug's final form [17,18]. A single method is never sufficient to resolve and characterize a protein. Multidimensional separation techniques using orthogonal separation modes with MS are also often unable to fully resolve all of the variants present within complex protein products [8].

The aim of this review is first to summarize the key steps in biopharmaceutical development for intact protein analysis. Then, the most widely used analytical methods and applications are highlighted, with particular emphasis on the electrophoretic and chromatographic separation techniques widely used in the biopharmaceutical field today.

2. Biopharmaceuticals: key steps in intact protein analysis

In this part of the review, the main stability issues and characteristics of therapeutic proteins as intact-molecule analysis are described. The corresponding analytical methodologies are further explained in related sections.

2.1. Characterization

Overall, proteins should be characterized in terms of identity, heterogeneity and impurity content. Proteins exhibit primary, secondary, tertiary, and, in some cases, quaternary structure. Characterization includes many parameters, such as molecular weight, size, isoelectric point, structure determination, purity assessment, charge state and charge microheterogeneity studies. Each property of the protein must be determined by at least two analytical strategies to ensure a coherent result. For example, the molecular weight can be calculated using size-exclusion chromatography, sodium dodecyl sulfate polyacrylamide gel electrophoresis, mass spectrometry (MS), light scattering and analytical ultracentrifugation. Since the underlying principles of these analytical methods are different, all results need to be corroborated to find a consensus value.

Proteins can also possess some post-translational modifications, such as phosphorylation, *N*- and *C*-terminal amino acid heterogeneity and glycosylation. The latter is the most common form of post-translational modification and consists of the enzyme-mediated process by which oligosaccharidic side chains are covalently attached to either the side chain of asparagine (N-linked) or serine/threonine (O-linked) [19]. The oligosaccharidic moieties of proteins are often essential for recognition, signaling and interaction events within and between cells and proteins, as well as for folding and defining protein conformation. Moreover, about 40% of approved therapeutics are glycoproteins [20]. Oligosaccharides are generally analyzed while still attached to the protein (peptide mapping and glycopeptide analysis), after intact releasing from the protein (oligosaccharide profiling) or after being broken down into their monosaccharidic constituent units (monosaccharide analysis). However, the analysis and characterization of intact proteins' glycoforms, such as recombinant erythropoietin [21], can also be achieved using mass analyzers with high or very high resolution (e.g., time-of-flight mass spectrometers (TOF/MS), Fourier-transform ion cyclotron resonance mass spectrometry (FT-ICR/MS) and Orbitrap).

2.2. Stability

Protein instabilities can be classified into two categories, chemical and physical. A recent review from Manning et al. [22] describes these phenomena. The resulting degradations can be implicated in causing adverse reactions like anaphylactoid reactions [23] and renal failure [24]. The manufacturing process consists of several operational steps, referred to as "unit operations", where the media is subjected to different stressors and conditions that could compromise quality and stability [25]. Instabilities can occur during

processing, handling, storage or use; it is therefore important to understand stability issues in all of these stages to successfully apply proteins as pharmaceuticals [26].

Chemical instabilities are due to processes that make (e.g., oxidation) or break (e.g., deamidation) covalent bonds, potentially generating new chemical entities [27]. Manning presents chemical instabilities into the following classes: deamidation, aspartate interconversion/isomerization, racemization, proteolysis, beta-elimination, oxidation, disulfide exchange, diketopiperazine formation, condensation reactions, pyroglutamate formation, hinge region hydrolysis and tryptophan hydrolysis. All of these reactions are dependent on numerous parameters, including pH, nature/concentration of excipients and temperature. Consequently, operating production conditions, purification, formulation and storage should be optimized and controlled to minimize chemical instabilities. Characterization of chemical degradation products is often performed by analyzing proteins in their intact form using liquid chromatography (LC), electrophoretic techniques or direct infusion or hyphenation with MS [28]. The combination of several analytical methods is often necessary to obtain a complete view of the degraded protein.

Physical instability is rarely encountered for low molecular weight molecules. Proteins, because of their polymeric nature and ability to form superstructures (e.g., secondary, tertiary and quaternary), can undergo a variety of structural changes independent of chemical modifications [29]. The principal types of physical instabilities are denaturation, surface adsorption, aggregation and precipitation. In particular, the control and analysis of protein aggregation is a growing challenge in pharmaceutical research and development [30]. Proteins may aggregate through several different mechanisms, classified as soluble/insoluble, covalent/non-covalent, reversible/irreversible, and native/denatured. Aggregates produced as a result of different stressors may have different size distributions, and their component proteins may contain different secondary and tertiary structures [31]. Protein aggregation can also lead to loss of activity, immunogenic reactions (e.g., in small aggregates) or adverse effects during administration (e.g., particulates) [32,33]. Several analytical methodologies for intact proteins are used for the determination of these physical degradations, such as multiangle light scattering, chromatographic and electrophoretic techniques, circular dichroism (CD), MS, and analytical ultracentrifugation [1]. As for chemical degradation, results obtained from several orthogonal analytical strategies are required to describe physical instabilities accurately.

3. Analytical methodologies

Analytical methodologies for intact protein analysis include chromatography, electrophoresis, mass spectrometry (MS) and spectroscopy. Particular attention has been paid to separation techniques such as chromatography and electrophoresis. Mass spectrometry and spectroscopy are mentioned with relevant references, but not in details. Hydrodynamic mass transport methods (e.g., viscosity, analytical ultracentrifugation) can also be used during pharmaceutical development, but these approaches are not discussed here.

3.1. Spectroscopy

Spectroscopy is commonly used to assess protein secondary and tertiary structure. Numerous techniques are available, such as X-ray, nuclear magnetic resonance (NMR), absorption, fluorescence, CD, dynamic light scattering (DLS), and infrared spectroscopy (IR) [10]. X-ray and NMR are often used to determine the three-dimensional structures of proteins, but present some limitations in routine implementation. For X-ray experiments, the proteins must be crystallized, which is particularly difficult for glycosylated proteins [6]. NMR is restricted to structures up to 25 kDa in size and often required protein concentrations higher than those used in the formulations. Absorption and fluorescence spectroscopy are used to describe the secondary structure of intact proteins (e.g., study of folding/unfolding). Absorption spectroscopy is also used to quantify proteins, according to the Bradford or Lowry methods. These approaches, which present an important lack of selectivity, are only capable of measuring an average signal change and cannot resolve contributions from individual amino acid residues. They provide indirect information on structure and conformation. CD spectroscopy is the technique of choice for studying chirality, particularly for monitoring and characterizing molecular recognition phenomena in solution [34]. It can also provide insights into the stereochemistry of a protein-bound drug or protein folding. Aggregates can also be analyzed by CD. Infrared spectroscopy can provide information about the protein secondary structure but involves complex spectra due to the number of atoms or groups of atoms in the protein. It is often used to complement CD methods, improving the estimation of secondary structure. DLS is primarily used to determine particle size and investigate protein aggregates [35].

Spectroscopy is widely employed in the pharmaceutical industry to elucidate structure and alteration during purification and formulation.

3.2. Mass spectrometry

Two fundamental strategies are used in the characterization and identification of proteins by MS. In the most commonly used "bottom–up approach", the mixture of proteins of interest is usually first digested by trypsin, and peptides generated are analyzed by MS and MS/MS. In the "top–down approach", intact protein molecular ions generated by electrospray (ESI) or matrix-assisted laser desorption (MALDI) are introduced into the mass analyzer [36]. The bottom–up strategy involves the analysis of peptides from the digested proteins, which is out of the scope of this review. The focus will thus be on the top–down approach only. The latter has the advantage of providing access to the complete protein sequence and the ability to locate and characterize post-translational modifications. However, in the case of ESI–MS, multiply charged ions are produced during ionization, generating complex mass spectra. Consequently, the method is often limited to isolated proteins or simple protein mixtures. In the context of protein development, this issue is often overcome since proteins are often isolated in pharmaceutical formulations. Thus, top–down MS is often preceded by a separation step (on-line or off-line), consisting of LC, capillary electrophoresis (CE) or other electrophoretic strategies [37]. In this context, mass spectrometers with high resolving power and accuracy should be used, such as TOF mass spectrometers, Orbitrap and FT-ICR instruments [38]. In biopharmaceutical development, intact protein analysis using the top–down approach should become more widely available, with Orbitrap instruments becoming less expensive to purchase and use compared to FT-ICR [39]. A milestone in protein characterization, MS provides precise and complementary structural information when used with other analytical techniques.

It has to be noted that two other MS approaches are also available: the Middle–Up and Middle–Down strategies. The Middle–Up one consists, as the Bottom–Up, in an enzymatic digestion but in bigger peptides, which are directly injected in the mass spectrometer. The Middle–Down mode uses the same peptides than the Middle–Up but they are fragmented in the mass spectrometer (MS/MS). The four approaches provide different information and are used in combination for the characterization of mAbs, for example.

3.3. Liquid chromatography

Liquid chromatography (LC) is recognized as an indispensable tool for intact protein analysis because of its high speed, high-resolving power, important reproducibility, and compatibility with MS [1]. Five different modes of LC have been employed for the analysis of intact proteins: reversed-phase LC (RPLC), size-exclusion chromatography (SEC), ion-exchange chromatography (IEC), hydrophilic interaction liquid chromatography (HILIC), and affinity chromatography (AC). Each mode comes with its advantages and limitations, which will be discussed in the upcoming sections, with comments about applications in biopharmaceutical analysis. A common aspect that should be evaluated for all modes is the adsorption of proteins onto the HPLC device. It is important to avoid the use of polyether ether ketone (PEEK) in connection tubings and injection needles. PEEK is a very hydrophobic material that causes strong protein adsorption. Inert materials such as titanium, stainless steel or PEEK-Sil (fused silica inside, PEEK outside) are preferable, although stainless steel and fused silica do not completely eliminate adsorption [40]. Recently, Agilent Technologies (Palo Alto, USA) introduced a new LC system (1260 Infinity Bio-inert HPLC Solution) dedicated to biomolecules analysis. The system is iron- and steel-free in solvent delivery and the sample contacting surface is completely metal-free, minimizing unwanted surface interactions. Moreover, Waters Corporation (Milford, USA) proposed a new UHPLC system (ACQUITY UPLC® H-Class Bio System), which also features an inert flow path and permits to perform four chromatographic modes on a single system (RPLC, IEC, SEC, and HILIC).

3.3.1. Reversed-phase liquid chromatography

In comparison to low molecular weight molecules and peptides, reversed-phase liquid chromatography (RPLC) of proteins is more problematic due to adsorption, carryover, lack of retention (pore exclusion), multiple peak formation, and low chromatographic performance. These issues arise because of slow diffusion through and secondary interactions with the stationary phase [41]. In addition, retention of proteins is strongly dependent on small changes in solvent strength, as recently reported by Gritti et al. [42]. For this reason, isocratic conditions are usually impractical. Even a change of 0.1% acetonitrile (ACN) would lead to a strong modification of protein retention, and percentage windows are quite narrow for each protein. Gradient elution is thus mandatory.

To improve RPLC on intact proteins, several approaches can be applied to reduce adsorption, minimize secondary interactions and enhance diffusion coefficients.

3.3.1.1. Reduction of protein adsorption. Adsorption of proteins onto the solid phase largely decreases sensitivity, due to analyte loss. This issue is particularly problematic when quantitation is to be performed or when small amounts of protein have to be analyzed. The first variable to consider modifying is the use of less hydrophobic stationary phases instead of conventional C_{18} phases [1]. Various types of sorbents (e.g., C_2, C_4, C_8, and C_{18}) are used in protein separation [43], but long-chain phases like C_8 or C_{18} can cause peak tailing or lower recovery of large protein due to their high hydrophobicity. Therefore, packing with shorter alkyl chain lengths like C_4 is preferable for proteins. However, this kind of chemistry is less resistant to hydrolysis in acidic pH conditions than C_8 or C_{18} phases. Adsorption issues with LC have been mentioned in Section 3.3. It is also worth noting that hydrophobic adsorption phenomena are partially overcome in RPLC due to the use of gradients to analyze proteins by LC. The increase in organic solvent composition with time could counteract and reduce this type of adsorption with numerous analyzed proteins.

3.3.1.2. Reduction of protein peak tailing. Secondary interactions occur between the positively charged analytes and the remaining negatively charged silanol groups of the stationary phase [44,45]. Because the kinetics of secondary ionic interactions are slower than that of hydrophobic interactions, chromatographic performance is reduced, resulting in peak tailing and broadening. To minimize secondary interactions, it is possible to use silica-based stationary phases with restricted access to residual silanols (e.g., endcapped, bidendate, hybrid silica, high density bonding, or embedded polar group stationary phase). Alternatively, the temperature of the mobile phase can be increased to enhance performance [46]. At elevated temperatures, mobile phase viscosity is reduced, analyte diffusivity is enhanced, and sorption kinetics are accelerated by improving mass transfer and kinetic rates [47]. Consequently, peak tailing and broadening are strongly reduced. However, temperature should be set with caution due to potential thermal degradation of proteins.

The third aspect to consider is the addition of an ion-pairing agent to the mobile phase. Trifluoroacetic acid (TFA) at a concentration of 0.1% is commonly used for protein analysis, as it possesses excellent ion-pairing and solvating characteristics and inhibits peak tailing and broadening [48]. However, when coupled with MS detection, ion suppression can occur with TFA, both in negative and positive modes. In this scenario, formic acid (FA) may be preferred. Finally, since some hybrid silica-based stationary phases are stable within a wide pH range (i.e., up to pH 12), it would be interesting to evaluate basic pH conditions to further reduce ionic interactions [49]. This option has only been reported for peptide analysis so far, but could certainly be extended to proteins [50,51]. Since peptides and proteins are multicharged molecules, comprised of mostly basic, ionizable functional groups, the change of mobile phase pH will have a pronounced effect on their retention behavior and peak shape.

3.3.1.3. Reduction of protein peak broadening. Conventional porous packing materials are well adapted for relatively small proteins and peptides obtained after proteolytic digestion. However, high-molecular-weight proteins have large radii and low diffusion coefficients that cause peak broadening when using conventional porous particles. Analyte diffusion in the pores significantly slows down as the pore size becomes smaller than approximately 10-fold the size of the analyzed compound. Therefore, larger porous packing materials with 300- or 1000-Å pore sizes have been introduced in place of the conventional 80–120 Å sizes. Numerous applications with these larger pore materials can be found in the literature [52–54]. An alternative approach is the use of elevated mobile phase temperature. As previously mentioned, temperature can be optimized to avoid peak tailing by accelerating the sorption kinetics of proteins. In addition, because diffusion coefficients are drastically enhanced with increasing temperature (i.e., Dm is proportional to the ratio of temperature/viscosity), chromatographic performance are improved. The use of high temperature (120 °C) LC was reported for the first time by Chen and Horvath in 1995, and a separation of four proteins in less than 10 s was successfully performed (Fig. 1) [46]. However, most stationary phases have limited thermal stability [55], and proteins are thermolabile molecules that can quickly be denaturated. For these reasons, high temperatures should be applied with caution.

One of the best solutions to limit band broadening in the case of intact protein analysis is the optimization of packing morphology. For this purpose, various solutions have been proposed. In the 1980–1990s, RPLC protein analysis was primarily carried out on non-porous sorbents [56]. The typical structure of non-porous stationary phases includes a fluid-impervious support physically or covalently attached to a layer of functional groups on its surface. A major advantage of this sorbent is that it decreases or avoids

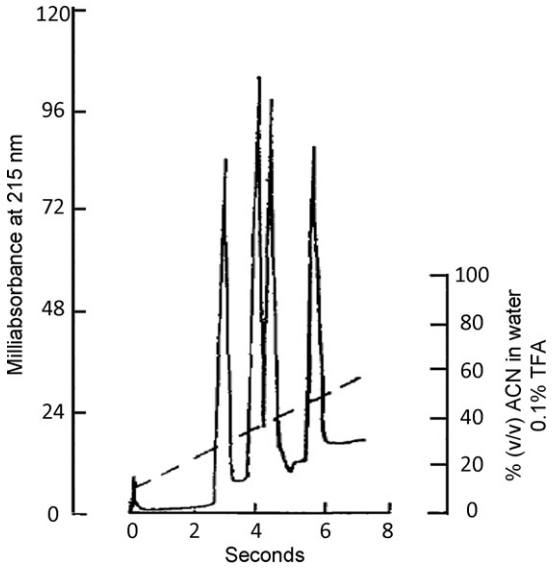

Fig. 1. Rapid separation of four proteins by RPLC (pellicular stationary phase) at elevated temperature (120°); 1, ribonuclease A; 2, cytochrome; 3, lysozyme; 4, β-lactoglobulin. Reprinted from [46] with permission from Elsevier B.V.

significant mass transfer resistances, resulting in improved analyte diffusion and high efficiency. However, the loading capacity is reduced proportional to the small specific surface area of the non-porous particles. Retention is also drastically decreased, which may hamper separation of most hydrophilic proteins. Porous layer open tubular (PLOT) columns that reduce mass transfer resistance have also been developed. They do not consist of a specific solid phase, but only a porous layer of stationary phase on the inner column wall, similar to a GC column. As non-porous supports, PLOT columns can provide high efficiencies for large molecules [57,58] with higher loading capacities. However, they may have some limitations in retention and selectivity, since the specific surface is not equivalent to that of sorbent particles.

Because of the inherent limitations of non-porous and PLOT materials, progress has also been made in optimizing the kinetic performance of packed columns with fully porous particles. Monolithic supports offer obvious kinetic advantages, including high permeability, low backpressure, and rapid mass transfer. These supports can be made with inorganic (e.g., silica, carbon, zirconia and titania) or organic (e.g., polymethacrylate and poly(styrene-divinylbenzene), polyacrylamide) materials. Organic monoliths are of limited interest for the separation of low molecular weight molecules, but useful for analyzing macromolecules because of their inert and biocompatible properties [59,60]. In the last two years, several applications have been published. Causon et al. studied the kinetic performance of a poly(styrene-divinylbenzene) monolithic column [61], and its use with elevated temperatures and alternative solvents [62]. They demonstrated some obvious advantages in cost, time, and reduction in organic solvent consumption. Eeltink et al. studied the same column and showed the interplay of the primary chromatographic parameters for the separation of intact proteins [63]. They highlighted the importance of column length and macropore size in achieving maximum peak capacity. In a previous work, they also employed such monoliths for one- and two-dimensional LC separations of intact proteins, reaching the highest possible resolution [64].

An alternative strategy consists of using columns packed with sub-2 μm particles. Reducing particle size leads to significant improvements in kinetic performance. This is particularly useful for large molecules, since optimal mobile phase flow-rate is inversely proportional to particle diameter and mass transfer resistance (i.e., C-term of the Van Deemter equation) is directly proportional to the square of d_p [65]. However, sub-2 μm supports generated high backpressure (>400 bar), which is not compatible with conventional LC instrumentation. Nevertheless, since the advent of commercial ultra-high pressure liquid chromatography systems (UHPLC) that withstand pressures up to 1300 bar, this issue can be counteracted. In 2008, Everley et al. used a combination of high temperatures (up to 65 °C), a strong organic modifier (i.e., isopropanol) and columns packed with sub-2 μm particles at very high pressure to yield enhanced resolution, sensitivity and a threefold increase in throughput for the analysis of 10 proteins [41]. Fig. 2 compares the original HPLC with the optimized UHPLC method, where enhanced resolution was obtained. However, when dealing with UHPLC conditions, it is important to keep in mind that pressure can have a strong influence on protein retention [66]. Indeed, when the linear velocity is increased between HPLC and UHPLC, the average column pressure is also significantly increased, and the retention factor could be strongly altered [42].

To increase the separation performance for large molecules, fused-core (also called core-shell or superficially porous particle) technology with 5-μm particles was originally introduced by Kirkland in 1992 [67]. It is now commercially available from numerous providers as sub-3 μm particles, allowing a significant improvement in kinetic performance. Compared to completely porous particles of similar sizes, the diffusion path is much shorter in fused-core technology because the inner core is solid fused silica, which is poorly penetrable by analytes [59]. For small molecules, it provides superior mass transfer kinetics and better performance at high mobile phase velocities [68] and lower backpressure [69]. On the other hand, Gritti et al. showed that the minimization of peak broadening at elevated linear velocities can be very attractive for intact proteins analysis [70].

3.3.1.4. Generic RPLC conditions and applications. To summarize, optimal RPLC conditions for intact protein analysis should consist of a compromise between efficiency/peak capacity, selectivity/retention, loading capacity, and protein adsorption. In this context, it seems suitable to employ UHPLC or fused-core technologies with a short alkyl chain length (C_4 phase) and a large pore size of 300 Å. The mobile phase temperature should be increased, and 0.1% TFA (with UV detection) or 0.1% FA (with MS detection) should be added to the mobile phase for their ion-pairing abilities.

In the biopharmaceutical field, RPLC appears to be promising for both the assessment of protein batch purity and to highlight any protein degradation (e.g., truncation, glycosylation, and isomerization) misfolds or PEGylation [7]. Due to recent advances in RPLC and its straightforward coupling to MS, its usefulness in intact protein analysis will become increasingly important.

3.3.2. Size-exclusion chromatography

Size-exclusion chromatography (SEC) is widely used for protein analysis because it is simple, robust, relatively high-throughput (compared to slab-gel electrophoresis), and readily available. A recent review by Arakawa et al. highlighted the importance of mobile phase composition (salt content, buffer concentration and organic solvent addition) on retention and recovery of proteins during SEC analysis [71]. On the other hand, significant drawbacks of SEC are its limited dynamic range, low efficiency and loading capacity, complex MS-coupling (few reported applications [1]), limited throughput, and protein adsorption onto the column. Diol-coated SEC columns packed with 1.7-μm particles (Waters Corporation, Milford, USA) were recently proposed to minimize secondary interactions, reduce column/system clogging and requirements for high-salt-concentration mobile phases, and significantly improve throughput. In the biopharmaceutical field, SEC is the method of choice for the characterization of aggregate size

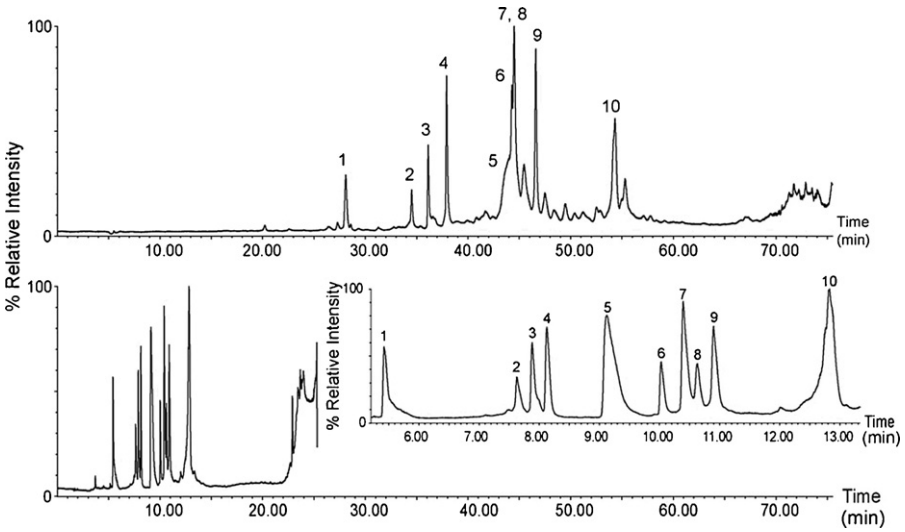

Fig. 2. Comparison of the original HPLC method (upper trace) and the optimized UHPLC method (lower trace) for the separation of 10 proteins. Reprinted from [41] with permission from Elsevier B.V.

and content [72], although large aggregates could not enter into the column due to clogging in the frits. Fig. 3 presents dual-wavelength SEC chromatograms for the IgG1 monoclonal antibody, exhibiting several aggregates. Finally, SEC is also widely used for protein purity and PEGylation determinations, ensuring batch-to-batch consistency.

3.3.3. Ion-exchange chromatography

Ion-exchange chromatography (IEC) is extensively used in protein separation, which usually involves many positive and negative charges. As with SEC, mobile phase composition is a critical because the IEC process strongly depends on salt concentration and pH. Cation-exchange chromatography is the gold standard for protein analysis because at a pH lower than 3, negative charges on carboxyl groups are neutralized, while N-terminus groups are protonated. Anion-exchange chromatography, which implies that carboxylic groups are negatively charged and N-terminus groups are neutralized, involves the use of high pH values (i.e., greater than 12) and is often incompatible with silica columns. Finally, IEC is often used in two-dimensional LC with hydrophobic interaction chromatography for the separation of native proteins [73]. In biopharmaceutical development, IEC remains the method of choice to analyze the charge heterogeneity of proteins and can be useful to determine PEGylations.

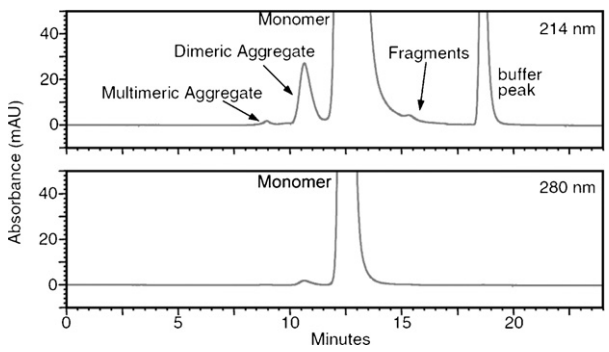

Fig. 3. Dual-wavelength size-exclusion chromatography for aggregate analysis of the IgG1 monoclonal antibody. Reprinted from [72] with permission from Wiley-VCH Verlag GmbH.

3.3.4. Hydrophilic interaction liquid chromatography

Hydrophilic interaction liquid chromatography (HILIC) is a variant of normal-phase chromatography in which the stationary phase is hydrophilic (i.e., bare silica or silica derivatized with various polar functional groups including amine, amide, cyano or diol), while the mobile phase consists of a mixture of water with more than 70% organic solvent (generally ACN). Retention is governed by different interactions including hydrogen bonding, hydrophilic partitioning between the stationary phase and the less polar mobile phase, and electrostatic interactions with charged groups at the surface of the stationary phase [74]. This technique is often used to analyze glycopeptides or glycans from glycoproteins [75]. HILIC has not yet been widely applied for the analysis of intact protein, aside from applications in the analysis of histones [76–78] and soluble proteins [79]. Recently, a paper describing online coupling of RPLC and HILIC for protein and glycoprotein characterization has been published [80]. In this work, the authors validated the applicability of their setup for the analysis of very complex biological samples. HILIC could be a valuable alternative to ion-exchange chromatography (e.g., by selecting an appropriate HILIC column with positive or negative charges at the surface), since it is directly compatible with MS instrumentation. Because HILIC columns packed with sub-2 μm or fused-core particles are now commercially available from several providers, interest in such chromatographic modes for intact protein analysis should quickly grow in the fields of protein characterization and stability studies. However, the possible irreversible adsorption of proteins onto the HILIC material still needs to be evaluated. Moreover, the dissolution solvent plays a major role in HILIC, and organic solvent solubility may be an issue for proteins, causing denaturation.

3.3.5. Affinity chromatography

Affinity chromatography (AC) is based on the interaction between target proteins and specific immobilized ligands. It creates either an enrichment or depletion of a specific class of proteins (e.g., highly abundant proteins in serum). AC can also separate proteins based on their biological activity, where an active form can be separated from the inactive one or a form with different biological function [81]. Interactions can occur through several entities: immunoglobulin (immunoaffinity), antibody fragments, bacterial proteins (protein A or G), lectins, or peptides [82]. Particularly, AC is widely used in the study of post-translational modifications, such as phosphorylation, glycosylation or cysteine oxidation–reduction.

3.4. Electrophoresis

Electro-driven separations are often used in the analysis of charged macromolecules. The principle is similar for all modes and consists of the separation of molecules under an electric field. Gel electrophoresis remains dominant, but capillary electrophoresis (CE) possesses some attractive characteristics, which will be highlighted in the next sections.

3.4.1. Gel electrophoresis

Fractionation by gel electrophoresis (polyacrylamide gel electrophoresis or PAGE) is based on sizes, shapes, and net charges of macromolecules. Systems designed to fractionate native proteins cannot distinguish between the impacts of size, shape, and charge on electrophoretic mobility. Consequently, proteins with different molecular weights could have the same mobility in these systems. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is an efficient strategy to overcome these limitations. SDS-PAGE imposes uniform hydrodynamic and charge characteristics on all proteins present in a sample mixture. Interactions with SDS disrupt all non-covalent protein bonds, causing the macromolecules to unfold. Electrophoretic mobility of the detergent–protein complexes is then related to molecular weight [83]. Detection is generally accomplished by staining with either Coomassie Brilliant Blue, or more sensitive silver stain dyes. However, SDS-PAGE requires extensive skill in gel pouring, sampling, separation, and staining/destaining for the visualization and evaluation of separated bands [84]. Moreover, very hydrophobic, extremely large, and highly basic proteins are often missing, and, finally, the reagents are quite toxic [85]. This approach is well suited for proteins with molecular masses higher than 10,000 Da with no post-translational modifications, where SDS-PAGE is commonly used to determine apparent molecular weight, size heterogeneity, purity, and manufacturing consistency [86].

Gel isoelectric focusing (IEF) methods are also often used for the characterization of therapeutic proteins (e.g., monoclonal antibodies). IEF is an electrophoretic separation method that separates amphoteric molecules like proteins according to their charge. The sample is prepared and conditioned with a chaotropic agent, a zwitterionic detergent, a reducing thiol, and carrier ampholytes to avoid the formation of aggregates and complexes between proteins. After the separation, when the proteins have reached their isoelectric point, gels are often incubated in SDS buffer to be subjected to SDS-PAGE as a second dimension. IEF can provide very high resolving power, including the separation of protein post-translational modifications that alter their charge (e.g., phosphorylation, acetylation) [87,88]. If desired, isoelectric points of proteins can be estimated with a calibration curve using marker proteins. The second dimension (SDS-PAGE) allows further separation based on the apparent molecular mass.

These methods are often used during the characterization step, due to their great resolving power and sensitivity. This is especially true for IEF because of the high concentration of proteins at their isoelectric point. Gel methods thus remain the gold standard among electro-driven techniques, although they are time-consuming and require good technical skills. They are also currently used as a preparative technique, where proteins of interest are excised from the gel prior to analysis by LC– or MALDI–MS [37].

3.4.2. Capillary electrophoresis

Capillary electrophoresis (CE) has several well-established, attractive features, such as high speed and great efficiency. Moreover, CE presents advantages of the capillary format (i.e., μL range): a smaller sample size, improved resolution, decreased separation time, full automation and real-time detection [89,90]. In the case of intact protein analysis, small differences between proteins may be sufficient for separation, since CE is a function of size, charge and shape. This method is promising for biopharmaceutical development because it is viewed as being orthogonal to RPLC and generally considered to be superior to classical electrophoresis.

Four modes of CE are commonly used for intact protein analysis: capillary gel electrophoresis (CGE), capillary isoelectric focusing (CIEF), capillary electrochromatography (CEC), and capillary zone electrophoresis (CZE). Micellar electrokinetic chromatography (MEKC) can also be used, but is less common due to its low efficiency for protein analysis [91]. MEKC and micro-emulsion electrokinetic chromatography (MEEKC) have been used in microfluidic chips to perform two-dimensional separations [92]. Affinity capillary electrophoresis (ACE) can also be used in intact protein analysis. ACE is particularly useful for studying biomolecular, non-covalent interactions and determining binding and dissociation constants of formed complexes. The review from Liu et al. summarizes these findings [93]. In following sections, we will focus on CGE, CIEF, CEC and CZE.

3.4.2.1. Adsorption issue.

The separation of intact proteins by CE is often hampered by their tendency to adsorb onto the negatively charged surface of conventional fused-silica-based capillaries. Adsorption can be reversible or irreversible, and both have a negative effect on CE separation performance. Reversible adsorption retards the migration time of proteins and decreases separation efficiency through adsorption/desorption events. Irreversible adsorption causes loss of proteins within the capillary and alteration of the electroosmotic flow (EOF) velocity. To monitor the reversible adsorption, peak efficiency and migration time relative standard deviation are recorded. EOF conservation and peak area recovery are likewise documented for irreversible adsorption [94]. Adsorption is influenced by the type of protein (e.g., flexible, rigid, isoelectric point), the pH and composition of the background electrolyte (BGE), the separation temperature, the nature of the solid surface, and the coating modification of the silica surface [95]. Minimizing protein adsorption is an important part of any optimization procedure, especially as the degree of adsorption to the capillary surface is not predictable from protein primary structure. Several strategies are possible to decrease protein adsorption: desorption by rinsing (e.g., NaOH, HCl or SDS solutions), electrolyte selection (e.g., phosphate buffers, addition of organic solvent in the BGE [96]), and capillary coatings. The latter is the most often used. Two types of coatings are available, dynamic and static. Dynamic coatings are versatile and cost effective, made by adding amines, surfactants or some neutral polymers directly to the BGE. Static coatings are made by permanently modifying the fused-silica surface through chemical reactions or physical adsorption of coating agents [97]. Choosing the type and nature of coating depends on both the detection system (e.g., static coatings are often preferred with MS detection to avoid serious background noise, suppression of analyte signal and/or contamination of ion source and MS optics) and the CE mode (e.g., in CIEF, no or low EOF is preferred with neutral or hydrophilic polymers). The adsorption issue could also be due to a protein used as an excipient. Lara-Quintanar et al. studied for example the immunochromatographic removal of albumin in erythropoietin biopharmaceutical formulations [98].

In all cases, evaluation of adsorption is a crucial step to use CE in the analysis of proteins. However, the potential adsorption surface is drastically reduced in CE compared to LC with packed material.

3.4.2.2. CE modes.

3.4.2.2.1. Capillary gel electrophoresis. Traditionally, SDS-PAGE has been used to monitor the size-based separation of proteins. As mentioned above, this technique presents some drawbacks including the use of toxic reagents and the low reproducibility

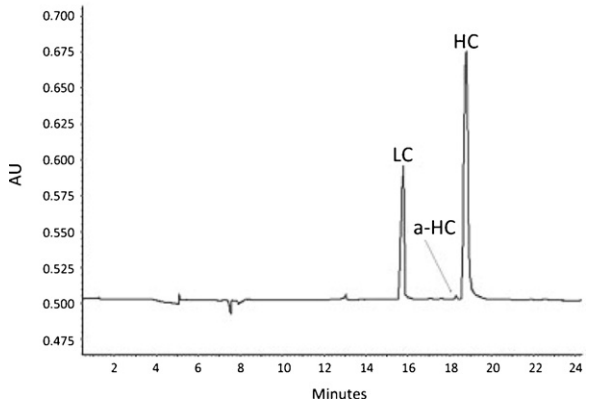

Fig. 4. Separation of a reduced IgG2 mAb using a mixed buffer matrix, which contained a dynamic coating (LC, light chain; HC, heavy chain; a-HC, agylcosylated heavy chain). Reprinted from [98] with permission from Wiley-VCH Verlag GmbH.

associated with the staining/destaining steps [84,85]. The fundamental principle of capillary gel electrophoresis (CGE) is similar to SDS-PAGE—samples are heated in the presence of SDS to denature the protein and impart a uniform negative charge. Proteins can be analyzed under their reduced or non-reduced form. The former is prepared by adding reducing agents such as β-mercaptoethanol, and the latter by adding an alkylating agent to prevent inter-chain disulfide bond shuffling during sample preparation. The sample is then injected into a capillary filled with a sieving gel comprised of a linear or branched polymer (e.g., polyacrylamide, PEG or dextran). The EOF must be strongly reduced by either a high buffer concentration, a buffer with a dynamic coating, or coated capillaries to achieve separation that is based solely on hydrodynamic radii as analytes migrate through the gel-sieving matrix [99]. CGE possesses some inherent advantages, such as automation, enhanced precision, high-speed analysis, improved resolution for closely migrating species, and online quantitative detection [100]. The high resolution of CGE is an advantage over SDS-PAGE and SEC. Over the past decade, CGE has been considered the primary method for size-based protein analysis, and its use has consequently grown in analytical and quality control laboratories, particularly for monoclonal antibodies (mAb) [101].

Recently, purity analysis of reduced and non-reduced IgG2 by CGE was developed, validated and implemented by Pfizer (Chesterfield, MO, USA) as a replacement for SDS-PAGE [99]. Fig. 4 shows the separation of a reduced IgG2 mAb using a mixed buffer matrix. The light and the heavy chains are perfectly separated. Guo et al. highlighted the structural isoforms of IgG2 in a non-reduced CGE method and demonstrated that both isoforms were disulfide bond-related species. Bioactivity studies of both isoforms have shown that they both possess the same potency [102]. In the development of targeted ultrasound contrast agents (UCA), monoclonal antibodies are often used due to their high specificity and affinity for the target (e.g., vascular molecular targets). The chemical coupling of UCA and mAb could have negative effects on mAb integrity. Cherkaoui et al. demonstrated that CGE-UV can be a powerful technique for monitoring IgG structural integrity under various reduction conditions [103]. CGE can also be used in miniaturized systems, as recently described by Wenz et al. They detected protein impurities down to a level of 0.05% relative to the main component using a microchip CGE and fluorescent derivatization [104]. This sensitivity could permit the routine use of miniaturized systems for purity and integrity analyses of biopharmaceuticals.

3.4.2.2.2. Capillary isoelectric focusing. Capillary isoelectric focusing (CIEF) combines the high resolving power and analyte concentration capacity of classical IEF with the advantages of the capillary format [105,106]. As in IEF, proteins are separated according to their isoelectric point (pI) in a pH gradient formed by carrier ampholytes when an electric field is applied [86]. CIEF has proven to be one of the most powerful methods in the analysis of protein isoforms, as it can distinguish between two proteins whose pI differs by as few as 0.005 pH units [107]. Coated fused-silica capillaries are usually used in CIEF to decrease EOF and reduce protein adsorption onto the capillary wall [108]. CIEF is commonly used to determine the pI of proteins, characterize impurities, and monitor protein charge heterogeneity (e.g., mAb). A great advantage of CIEF is its compatibility with MS detection. CIEF coupled online with MS is a promising alternative to 2D-PAGE, since it is also a 2D-separation [109]. Offline coupling with MALDI/MS was performed by Minarik et al. by using a fraction collection interface and analyzing a mix of standard proteins [110]. This setup was also used in 2009 to characterize glucagon and its deamidation product [109]. The online coupling of CIEF with MS via an electrospray ionization (ESI) interface is also promising, but the presence of carrier ampholytes and polymeric additives could interfere with protein ionization (i.e., ion suppression). To overcome this limitation, relatively low concentrations of carrier ampholytes should be used [111]. Mokaddem et al. proposed an online coupling of CIEF and ESI/MS in a glycerol–water media [112]. Glycerol was a good alternative to conventional aqueous gels for CIEF separations, since the presence of gel in the separation medium is a major constraint in CIEF–ESI–MS. Since glycerol strongly reduced EOF, no capillary coating was needed. The procedure may allow the characterization of both hydrophilic and hydrophobic proteins in samples of intermediate complexity. Fig. 5 shows data for the scan-mode MS signal, single ion monitoring (SIM) signal and extracted ion current (EIC) chromatogram of six model proteins obtained with sufficient resolving power.

A recent review by Shimura highlighted advances in CIEF with microchips [113]. Overall, automation and separation resolution should be improved before this technique can be widely used.

3.4.2.2.3. Capillary electrochromatography. Capillary electrochromatography (CEC) is a hybrid technique where the separation between analytes results from a combination of electrophoretic migration and chromatographic retention. Consequently, advantages of CE (i.e., the capillary format, low consumption of solvents, high efficiency, and flat flow profile) are theoretically achieved along with those of LC (i.e., elevated selectivity and possible separation of neutral molecules). However, some limitations are encountered in CEC such as poor robustness, low sample capacity, and insufficient reproducibility. There are three modes of CEC: columns (capillaries) packed with porous particles, columns with monolithic materials, and open-tubular systems. Reviews published by Oliva et al. [1] and Miksik et al. [114] extensively described these different strategies. The traditional approach uses columns packed with particular chromatographic materials. For protein separation, RP and ion-exchange materials are typically used. Zhang et al. proposed a CEC separation of four standard proteins with a strong anion-exchange column [115]. Columns made of a cationic acrylic monolith have been used by Zhang et al. to separate a mixture of standard proteins and peptides [116]. They highlighted the complex interplay between selective chromatographic retention and differential electrophoretic migration. The use of open-tubular systems requires a coating to avoid protein adsorption. Recently, Moore et al. developed a zwitterionic coating with both carboxylic acid and amine groups. It creates either an overall positively or negatively charged coating, depending on BGE pH [117]. Microfluidic devices can also be used in CEC, although development has been limited due in part to the difficulty of packing microfluidic networks with stationary phase materials. In 2009, Jemere et al. presented a baseline separation

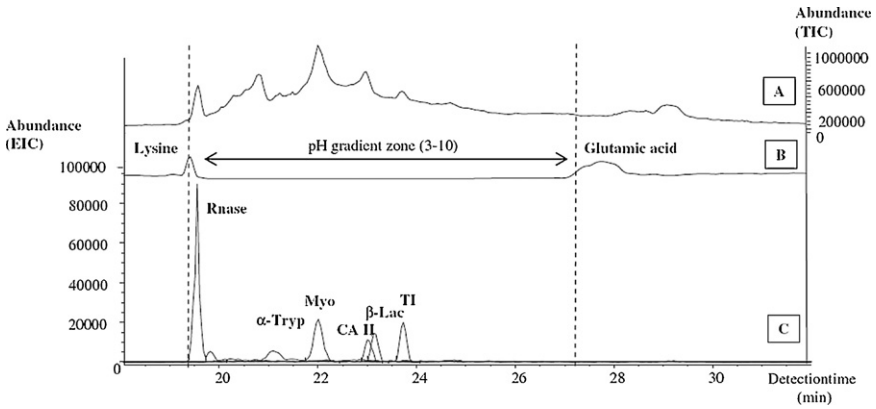

Fig. 5. CIEF–ESI/MS and extracted MS spectra of six model proteins: (A) total ionic current electropherogram; (B) single ion monitoring electropherogram; (C) extracted ion chromatogram of Rnase (ribonuclease A), α-Tryp (α-chymotrypsinogen), Myo (myoglobin), CA II (carbonic anhydrase II), β-Lac (β-lactoglobulin), and TI (trypsin inhibitor). Reprinted from [111] with permission from Wiley-VCH Verlag GmbH.

of labeled insulin and immunoglobulin G with microchip-based bead-packed columns [118].

CEC can also be coupled to MS detection [119]. The most common interfaces are electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI). CEC-MS is commonly used for the analysis of amino acids, peptides, saccharides, and chiral compounds, among others. To the best of our knowledge, intact protein analysis by CEC-MS has not yet been reported.

The potential of CEC in protein analysis is still negligible due to the lack of stationary phases specially designed for protein separation [120]. Combined with the issues associated with CEC, it is clear why there are only a small number of applications in biopharmaceutical protein analysis [121].

3.4.2.2.4. Capillary zone electrophoresis. In capillary zone electrophoresis (CZE), the capillary is generally filled with a background electrolyte (BGE) and separation is accomplished by differences in the analytes' electrophoretic mobility [89]. High separation efficiencies can be obtained, with longitudinal diffusion being the only source of band broadening. In CZE, efficiency is inversely proportional to the diffusion coefficient of molecules. This is particularly attractive for intact protein analysis, since these compounds have low diffusion coefficients. CZE is the most frequently used mode that can be hyphenated with MS for intact protein analysis. Since the BGE can be made of only volatile components, it is directly compatible with MS, commonly through an ESI interface. MS compatibility provides useful structural information, particularly when coupling with high resolution mass spectrometers [89,122]. The study of adsorption is crucial in CZE, since fused-silica capillaries are generally used with a BGE containing only buffer. As explained previously, the use of capillary coatings (dynamic or static) is the best approach to counteract protein adsorption onto the capillary wall. Catai et al. proposed for example the use of non-covalently bilayer-coated capillaries for intact proteins analysis [123–125]. In biopharmaceutical protein research, CZE is now often used to identify the therapeutic mAb, and examine its charge heterogeneity. He et al. developed a CZE method in a 40-cm, uncoated capillary for the separation of IgG1 and IgG2 monoclonal antibodies [126]. Charge variants were separated with a short capillary (10 cm effective length). CZE can also be applied to the identification of protein isoforms. Bohoyo et al. used CZE in the analysis of different isoforms of unphosphorylated recombinant tau protein and for the separation of the phosphorylated and unphosphorylated protein forms [127]. A polybrene coating was used to reduce adsorption of the tau protein, which presents numerous cationic moieties that interact strongly with the capillary wall. Balaguer et al. characterized the glycoforms of erythropoietin by combining glycan and intact protein analysis using CE and time-of-flight MS [128]. The molecular masses and the quantitation of each intact glycoform were determined. Fig. 6 presents the mass spectrum and corresponding deconvoluted mass spectrum of the analyzed recombinant human erythropoietin. The spectrum obtained is relatively simple, and, as

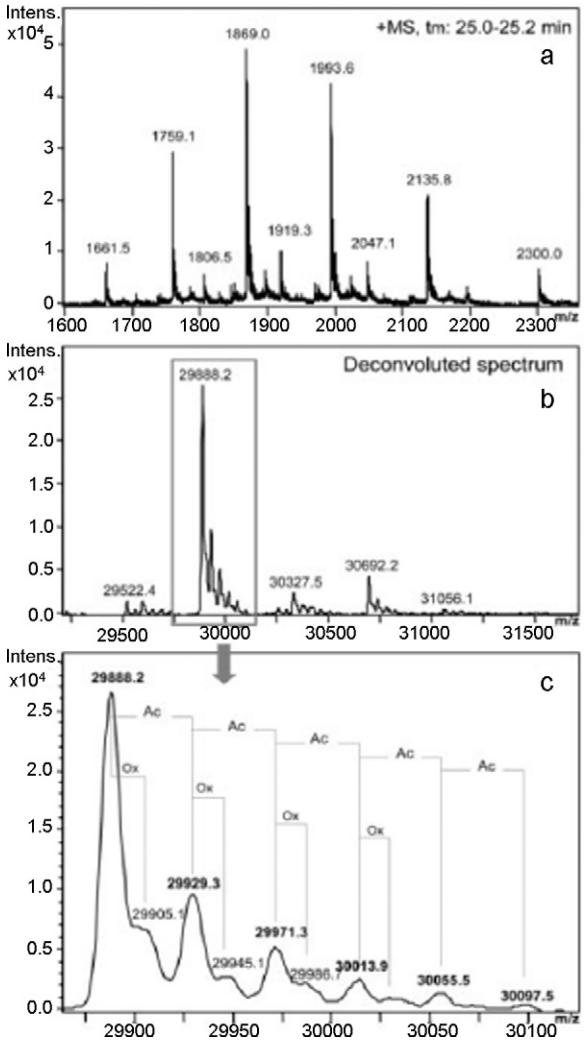

Fig. 6. CE-TOF/MS mass spectrum (a) from separation of intact recombinant human erythropoietin, (b) the deconvoluted mass spectrum, and (c) details of the deconvoluted spectra (Ac, acetylation; Ox, oxidation). Reprinted from [127] with permission from Wiley-VCH Verlag GmbH.

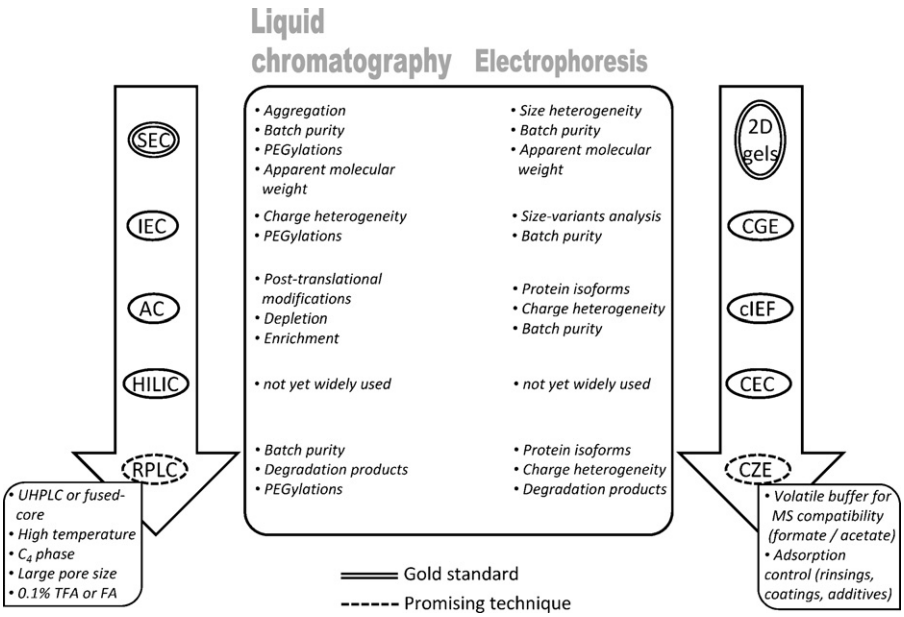

Fig. 7. Separation techniques described in this review and their applications.

shown in Fig. 6b, a charge envelope corresponding primarily to one glycoform is obtained. Berkowitz et al. developed a CZE method to detect various forms of oligosaccharides and the amount of deamidation on the glycoprotein [129].

CZE is particularly well adapted for intact protein analysis during development (e.g., for quality control), where simple and efficient analytical methods are required. In this context, Staub et al. presented the analysis of human growth hormone seized samples [130] and identification and quantification of insulin formulations using CE and a time-of-flight mass analyzer [131]. Furthermore, CZE methods were introduced in the European Pharmacopeia for the analysis of human growth hormone [132] and erythropoietin [133]. Some improvements were then proposed and published. For example, Catai et al. introduced a coating step to improve the repeatability of human growth hormone analysis [125]. Zhang et al. selected an alternative protein standard as reference material and modified the capillary conditioning for erythropoietin analysis [134].

3.4.2.3. CE perspective. Every pharmaceutical protein either on the market or in development has been characterized by electro-driven approaches. CE is now recognized by pharmacopeias and extensively used for quality control by companies in the context of lot release, product development, recovery, process design, formulation and stability analyses [90]. Microfluidic CE devices for proteins show promises to increase the contribution of CE to this area. Development of miniaturized CE systems for protein analysis has advanced tremendously in recent years, and significant progress has been made in terms of EOF control and limits of detection [135]. Improvements in detection, reproducibility and ease of fabrication will provide solutions for the analysis of new biopharmaceutical drugs, particularly in the context of onsite analysis.

4. Conclusion

Over the last decade, the number of pharmaceutical proteins in development and in the market has become more significant. This is principally due to the advances made in the field of biotechnology. These new products are much more difficult to analyze than "classical" drugs resulting from chemical synthesis (e.g., microheterogeneity, numerous molecular weights, possible conformations, and post-translational modifications). Their complexity necessitates the development of new analytical strategies to characterize and ensure the safety of these biopharmaceuticals. Physical and chemical stabilities also have to be studied. If chemical instabilities are well known for low molecular weight chemical molecules, physical instabilities will be more specific for proteins. To obtain a comprehensive picture of a protein in terms of its structure, conformation, post-translational modifications and stability, numerous analytical strategies with different principles are needed. In this review, emphasis has been put on intact protein analysis and separation techniques. Fig. 7 summarizes the separation methods described in this review, highlighting the gold standards and promising techniques, as well as applications for each analytical technique. Liquid chromatography is already well established in industrial laboratories for intact protein analysis (e.g., size-exclusion, ion-exchange chromatography). However, recent technological developments of RPLC stationary phases for the analysis of intact proteins (i.e., sub-2 μm or fused-core particles with short alkyl chain lengths, C_4, and large pore sizes of 300 Å) make this a promising technique. Gel electrophoretic approaches remain the gold standard for apparent molecular weight, size heterogeneity, purity, and manufacture consistency determinations, although they are time-consuming and need good technical skills. Capillary electrophoresis is also commonly used in the biopharmaceutical industry. Specifically, capillary gel electrophoresis and capillary isoelectric focusing modes permit the combination of the high resolution of gel techniques and the advantages of the microfluidic format of capillaries. In this context, capillary electrophoretic techniques could partially substitute future gel electrophoretic methods. Capillary zone electrophoresis appears to be a good candidate, since its easy coupling with time-of-flight mass spectrometry could provide important information with simple and efficient analytical methodology. Mass spectrometry (top–down approach) and spectroscopy are also widely used to collect complementary structural information regarding 2D and 3D protein conformation. Overall, several analytical approaches are always needed to cover all protein properties. Recent technological progress will contribute to a better knowledge of these parameters and help to understand the impact of changes in manufacturing on the quality and consistency of biopharmaceutical drugs.

Conflict of interest

The authors have declared no conflict of interest.

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