Analytical strategies for the characterization of therapeutic monoclonal antibodies

Szabolcs Fekete, Anne-Laure Gassner, Serge Rudaz, Julie Schappler, Davy Guillarme

Recombinant monoclonal antibodies (mAbs) have become particularly relevant for the treatment of autoimmune diseases or cancers. Because of their inherent complexity and for safety reasons, there is a need to develop powerful analytical methods to provide detailed characterizations of mAbs.

The aim of the present review is to detail the state-of-the-art of analytical strategies for mAb characterization. It focuses on the most important separation techniques used in this field, specifically, the chromatographic and electrophoretic approaches and their combination with mass spectrometry (MS). Thanks to recent improvements in separation science and MS devices, mAbs can be analyzed more easily. However, there is still a need to find new approaches that avoid adsorption issues.
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Abbreviations: BGE, Background electrolyte; CD, circular dichroism; CIEF, capillary isoelectric focusing; CZE, Capillary zone electrophoresis; EMA, European Medicines Agency; EOF, Electroosmotic flow; ESI, Electrospray ionization; Fab, Fraction antigen-binding; Fc, Fraction crystallizable; FDA, Food and Drug Administration; FL, Fluorescence spectrophotometry; FT-IR, Fourier transform infrared spectroscopy; HC, Heavy chain; ICH, International Conference on Harmonisation; IEX, Ion exchange chromatography; Ig, Immunoglobulin; LC, Light chain; LOD, Limit of detection; LOQ, Limit of quantification; LP, Limited proteolysis; mAb, Monoclonal antibody; MALDI, Matrix-assisted laser desorption/ionization; MS, Mass spectrometry; NGHC, Non-glycosylated heavy chain; PLOT, Porous layer open tubular; RPLC, Reversed-phase liquid chromatography; RSD, Relative standard deviation; SDS-PAGE, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SEC, Size exclusion chromatography; UHPLC, Ultra-high-pressure liquid chromatography

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1. Introduction

1.1. The therapeutic mAb market

Monoclonal antibodies (mAbs) are an emerging class of therapeutic agents currently being developed by many pharmaceutical companies. In 2010, the global therapeutic mAb market was a $48 billion ($40 billion and $37 billion for 2009 and 2008, respectively) [1], which represents approximately 5% of the pharmaceutical market, estimated at $850 billion.

Cancers of the colon, breast, lung, head, and neck and arthritis accounted for over 75% of the total mAb market, but new molecules introduced to treat chronic illnesses (e.g., asthma or osteoporosis) could further expand the mAb market. Muromonab-CD3, developed to reduce acute rejection in organ-transplant patients, was the first therapeutic mAb approved for use in humans in 1986. Since 2007, approximately 40 novel mAbs have begun clinical studies each year, and new products are regularly approved by the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA) [2]. The FDA has now authorized more than 20 mAbs, and 150 other mAbs, primarily involving immunological and oncological targets, are currently undergoing clinical trials with an approval rate of approximately 20% compared with 5% for new chemical entities [3].

Several characteristics of mAb therapy contribute to its success by improving the risk-benefit ratio. These characteristics include improved tolerance, good efficacy,

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high specificity, and limited side effects. Furthermore, the difficulty in obtaining generics (biosimilars or follow-on biologics) makes mAbs more attractive to patent holders than small molecules [4].

1.2. mAbs structure

The mAbs are glycoproteins that belong to the immunoglobulin (Ig) superfamily, which can be divided into five isotypes: IgA, IgD, IgE, IgG, and IgM. Because only IgGs are produced for therapeutic purposes through genetic engineering, the terms recombinant mAb and IgG are often used interchangeably.

The general structure of a mAb and its fragments is illustrated in Fig. 1. IgGs are large tetrameric glycoproteins measuring approximately 150 kDa that are structurally composed of four polypeptide chains: two identical heavy chains (HC, ~50 kDa) and two identical light chains (LC, ~25 kDa), connected through several inter-chain and intra-chain disulfide bonds at their hinge region. The resulting tetramer has two similar halves, forming Y-like shapes [5]. Each chain is composed of structural domains according to their size and function, giving the constant, variable, and hypervariable regions. Differences between the HC constant domains are responsible for the IgG sub-classes (i.e. IgG1, IgG2, IgG3, and IgG4).

Functionally, mAbs consist of two regions: the crystallizable fraction (Fc) and the antigen-binding fraction (Fab) [6]. As shown in Fig. 1, Fc (~50 kDa) is composed of two truncated HCs and is responsible for the effector functions (e.g., complement fixation and receptor binding). The Fc sequence also has a conserved N-glycosylation site, which is generally occupied by a biantennary oligosaccharide accounting for significant effects on the activity and efficacy of the IgGs [7]. The Fab domain (~50 kDa) is composed of the LC and the remaining portion of the HC. This domain is primarily involved in antigen binding [6].

Because mAbs exhibit great molecular complexity, they may be quite sensitive to changes in the manufacturing processes that can lead to considerable micro-heterogeneity in each individual chain. There are several common modifications leading to antibody-charge variants (or isoforms) on the peptide chains (e.g., deamidation, C-terminal lysine truncation, N-terminal pyroglutamation, methionine oxidation, or glycosylation variants) and size variants on the peptide chains (e.g., aggregation or incomplete formation of disulfide bridges). The combination of these micro-heterogeneity sources in the peptide chains significantly increases the overall heterogeneity in an entire IgG.

1.3. mAb characterization

Due to the increasing number of approved mAbs in the pharmaceutical area and the number of biosimilars potentially entering the market, the need for analytical techniques adapted for their detailed characterization has increased. As previously discussed, the intrinsic micro-heterogeneity is of major concern with mAbs and should be critically evaluated because differences in impurities and/or degradation products could lead to serious health implications [8].

The complete characterization of an intact mAb is difficult to achieve, so various enzymes (e.g., pepsin, papain, and Lys-C) are often used to obtain mAb fragments and facilitate the investigation of its micro-heterogeneity. As illustrated in Fig. 1, papain is primarily used to cleave IgGs into three fragments at the HC hinge region, one Fc and two identical Fab fragments of ~50 kDa each, while pepsin generates $F(ab')_2$ fragments measuring ~100 kDa. These types of digestion are called limited proteolysis (LP). The reduction of disulfide bonds is also commonly used to produce 2 LCs and 2 HCs.

In general, identity, heterogeneity, impurity content and activity of each new batch of mAbs should be thoroughly investigated before release. This examination is achieved using a wide range of analytical methods, including reversed-phase liquid chromatography (RPLC), size-exclusion chromatography (SEC), ion-exchange chromatography (IEX), sodium-dodecyl sulfate polyacrylamide-gel electrophoresis (SDS-PAGE), capillary-isoelectric focusing (CIEF), capillary zone electrophoresis (CZE), circular dichroism (CD), Fourier transform infrared spectroscopy (FT-IR), fluorescence spectrophotometry (FL), and mass spectrometry (MS). The goal of this multi-method strategy is to demonstrate the similarity between production batches of mAb by precisely determining the primary, secondary, and tertiary structures of the mAbs [9]. The chromatographic and capillary electrophoretic methods described in this review are particularly well suited to this purpose.

2. Chromatographic approaches

2.1. Size-exclusion chromatography (SEC)

Size distribution or mono-dispersity of a mAb product is important for both safety and efficacy. Components smaller than the intact mAb are often the result of enzymatic or non-enzymatic cleavage and the incomplete formation of mismatched disulfide bridges. In the latter case, neither the light chain nor the heavy chain is entirely incorporated into the IgG molecule. The size-related heterogeneity due to components larger than the individual antibody is often a result of molecular association, aggregation, or even precipitation. A full spectrum of species, from molecular dimer to oligomer to higher-order aggregates, may be present in a mAb preparation [10].

SEC is commonly used to determine size-related heterogeneity. SEC separations can be applied to both native and denatured antibodies for assessing whether the association is covalent or non-covalent. SEC is also

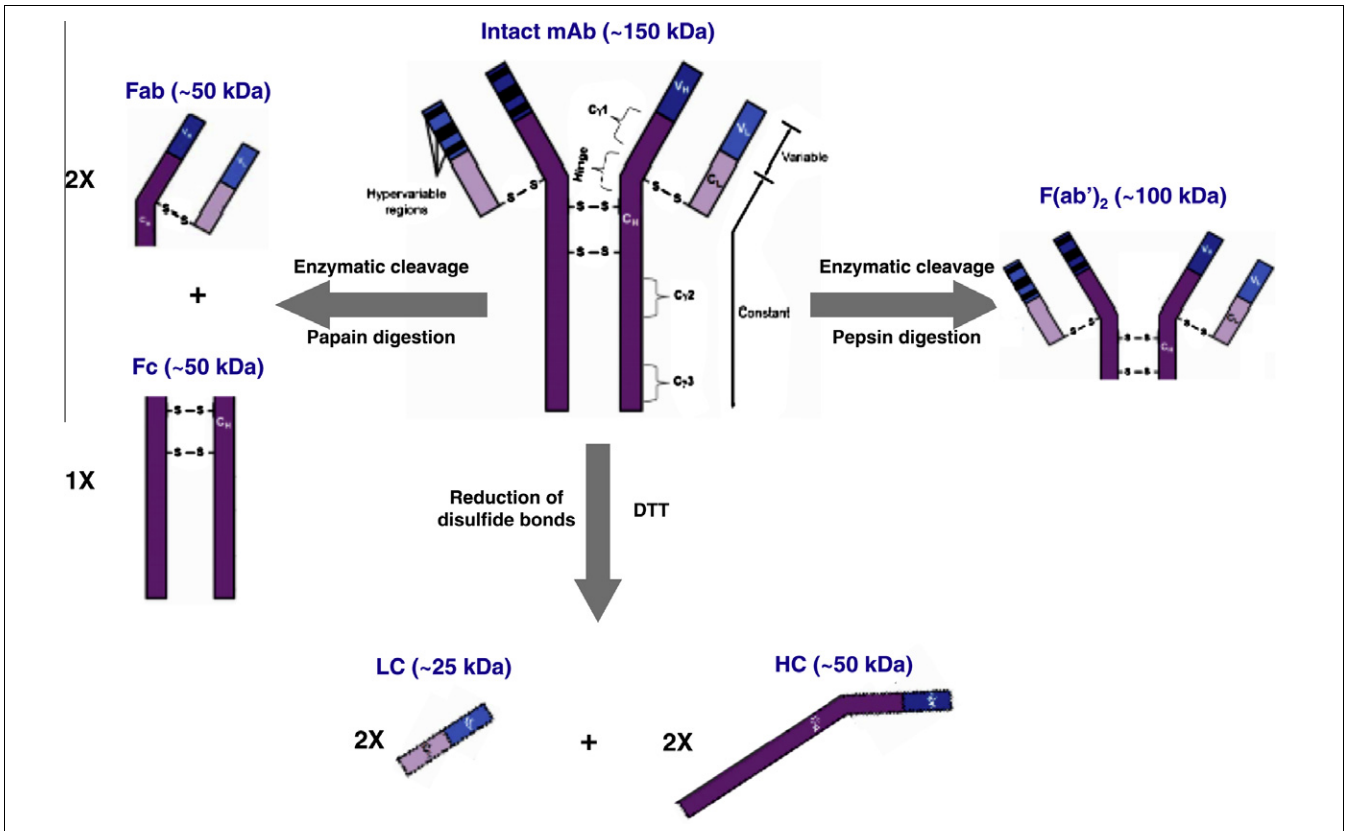


Figure 1. The general structure of a monoclonal antibody and its fragments. LC, Light chain; HC, Heavy chain; Fab, Fraction antigen-binding; Fc: Fraction crystallizable.

useful in determining if cleaved components are incorporated into monomeric molecules under native conditions [10]. SEC separates biomolecules according to their molecular size. This technique uses a gel suspended in an aqueous buffer solution that is packed into a chromatographic column. The gel consists of spherical porous particles with a carefully-controlled pore size, through which the biomolecules diffuse based on their molecular size differences. The separation power of an SEC column increases in direct proportion to the square root of the column length, so the separation of complex samples requires long columns that can be obtained by joining multiple columns in a series.

An interesting study showed that mAbs can produce low levels of Fab and Fab + Fc fragments upon extended incubation in the liquid state. The fragments were perfectly separated and identified by SEC [11].

A recent trend in SEC is the reduction of column dimensions (length and internal diameter) and particle size to improve peak width, as demonstrated by RPLC that uses sub-2 μm particles. SEC columns with a length of 15 cm and packed with 1.7-μm particles are now commercially available. With efficient stationary-phase geometry, the time required for the separation of mAb aggregates and fragments can be reduced to 5–10 min, as shown in the application notes from the column providers. However, reducing the particle size to the sub-2 μm range can increase the risk of shear degradations [12].

2.2. Ion-exchange chromatography (IEX)

In IEX, charge variants are separated by differential interactions on a charged support. The number of possible charge variants increases with the molecular weight. In addition, changes in charge may be additive or subtractive, depending on any modifications. Thus, IEX profiles become more complex, and the overall resolution of individual variants may be lost. This property is particularly apparent for mAbs with molecular weights of ~150 kDa, so both the so-called limited proteolysis approach (producing Fab and Fc fragments) and the analysis of intact mAbs are commonly used in IEX.

Moorhouse et al. previously described the potential of IEX for mAb analysis [13]. Papain-digested mAb samples were separated with sufficient resolution and their fragments identified with MS (Fig. 2). The C-terminal lysine variability of the Fc and the N-terminal glutamine-pyroglutamate variability of the Fab were observed.

A recent study demonstrated the suitability of IEX for studying complex degradation processes involving various IgG1 molecules [14]. Assignment of covalent degradations to specific regions of the mAbs was facilitated using Lys-C and papain to generate Fab and Fc

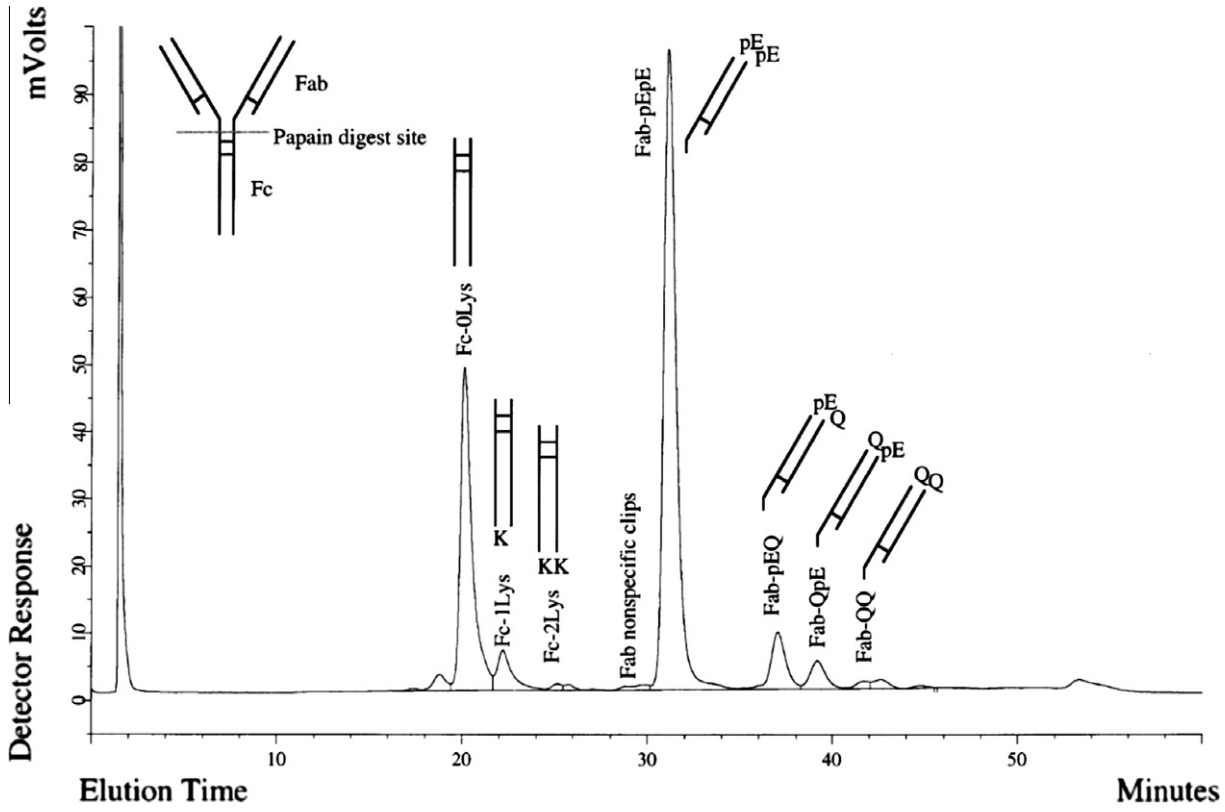


Figure 2. Ion-exchange chromatographic separation of the Fab and Fc variants from a papain-digested mAb sample. Column: PL-SXC, the gradient program was 5 min at 95% solvent A, 5% solvent B (0.2 M NaCl in 10 mM MES pH 6.0) followed by a linear gradient of 5–85% solvent B over 40 min (flow rate 1 ml/min). The column temperature was ambient and elution was monitored at 280 nm. The C-terminal lysine (K) variants of Fc and the N-terminal glutamine (Q)-pyroglutamate (pE) variants of Fab are well resolved (from [13] with permission).

fragments. This method was particularly useful for characterizing protein variants formed in the presence of salts under accelerated storage conditions. Two isoforms accumulated during storage were readily identified as Fab-related species. The usefulness of this assay was further illustrated by characterization of light-induced degradations of mAb formulations.

Another study presented the importance of IEX in the analysis of oxidized mAb samples [15]. Both cation-exchange and anion-exchange chromatography were used and found suitable for the separation of the most basic oxidized variants of the intact mAbs. Similar to SEC, manufacturers recently launched more efficient IEX columns packed with relatively small (5–7 μm) or non-porous particles.

2.3. Reversed-phase liquid chromatography (RPLC) and RPLC-MS

2.3.1. Conventional RPLC and RPLC-MS approaches for mAb analysis. In RPLC, solute retention is predominantly mediated through the hydrophobic interactions between the non-polar amino-acid residues of the mAbs and the bonded n-alkyl ligands. For RPLC analysis, the gradient-elution mode is preferred and the solutes are therefore eluted in order of increasing hydrophobicity. The retention of mAbs strongly depends on minor variations in the solvent strength; a small change (<1%) in the organic modifier content can lead to a significant retention shift. For this reason, isocratic conditions are impractical, and gradient elution is necessary.

A bioanalytical assay using RPLC coupled with fluorescence detection was developed by Damen et al. for the bioanalysis of the mAb trastuzumab after immunoaffinity purification [26]. Trastuzumab was isolated from human serum by a sepharose material coupled with anti-trastuzumab idiotype antibodies. After extraction, the samples were injected onto a Zorbax 300SB C8 column at 75°C using a mobile phase containing both isopropanol and acetonitrile with a high eluotropic strength.

Its potential compatibility with MS is an obvious advantage of RPLC over other chromatographic approaches for mAb characterization (*e.g.*, IEX, SEC, and affinity chromatography). This detection technique has become increasingly popular for the characterization of proteins thanks to:

(1) the introduction of two ''soft'' ionization techniques that enable the transfer of intact proteins into the gas phase without fragmentation [i.e. electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI)]; and,

(2) the continuous improvements of MS analyzers that provide high mass resolution, high mass range, and high sensitivity detection (e.g., TOF/MS and Orbitrap).

Complete proteolytic digestion of a mAb (peptide mapping) followed by gradient RPLC-MS/MS analysis (''bottom-up'' approach) is generally the method of choice for identification and quantification of chemical modifications of mAbs [16,17]. However, this approach is time consuming and can induce modifications during the relatively complex sample preparation [17]. By contrast, the analysis of intact or fragmented mAbs requires minimal sample preparation and can provide a high-throughput alternative to peptide mapping. For these reasons and through advances in RPLC columns and instrumentation, the second approach is currently preferred over traditional peptide mapping.

Using RPLC without enzyme digestion and under reducing conditions, LC can produce a single sharp peak, but HC fragments often elute in a more heterogeneous peak with slightly resolved forms. Significantly increasing the mobile phase temperature improves the resolution [8]. However, this process may lead to cleavage of the acid-labile asparagine-proline bond in the heavy chain, so, without enzyme digestion, the use of conventional RPLC is limited and has been employed exclusively for confirming the overall purity and the integrity of LC and HC under denaturing conditions [8].

Limited proteolysis is also used prior to mAb analysis by RPLC. Several recent publications have described the analysis of IgG1 Fab and Fc domains using RPLC and RPLC-TOF/MS to confirm chemical and post-translational modifications (e.g., N-terminal cyclization, oxidation, deamidation, and C-terminal processed lysine residues) [18,19]. In another example, an enhanced analytical RPLC-MS method was developed to monitor the stability and the production of intact and fragmented mAbs [20]. The use of elevated column temperatures (70–80°C) and organic solvents of high eluotropic strength (isopropyl and *n*-propyl alcohols) provided good protein recovery (limited adsorption) and resolution of the IgG1 and IgG2. Using this method, cleavage products of a degraded IgG1 antibody were clearly separated and identified by on-line ESI-TOF/MS generating exact masses for the unique terminal-ladder sequences.

An accurate method to detect tryptophan oxidation on the HC was also developed [21]. Fig. 3 shows the chromatograms of the reduced mAb. As depicted, the oxidized forms of HC can be separated with sufficient resolution.

A new combinatory strategy to analyze the heterogeneity of the LC, the single chain Fc (sFc), and the Fab

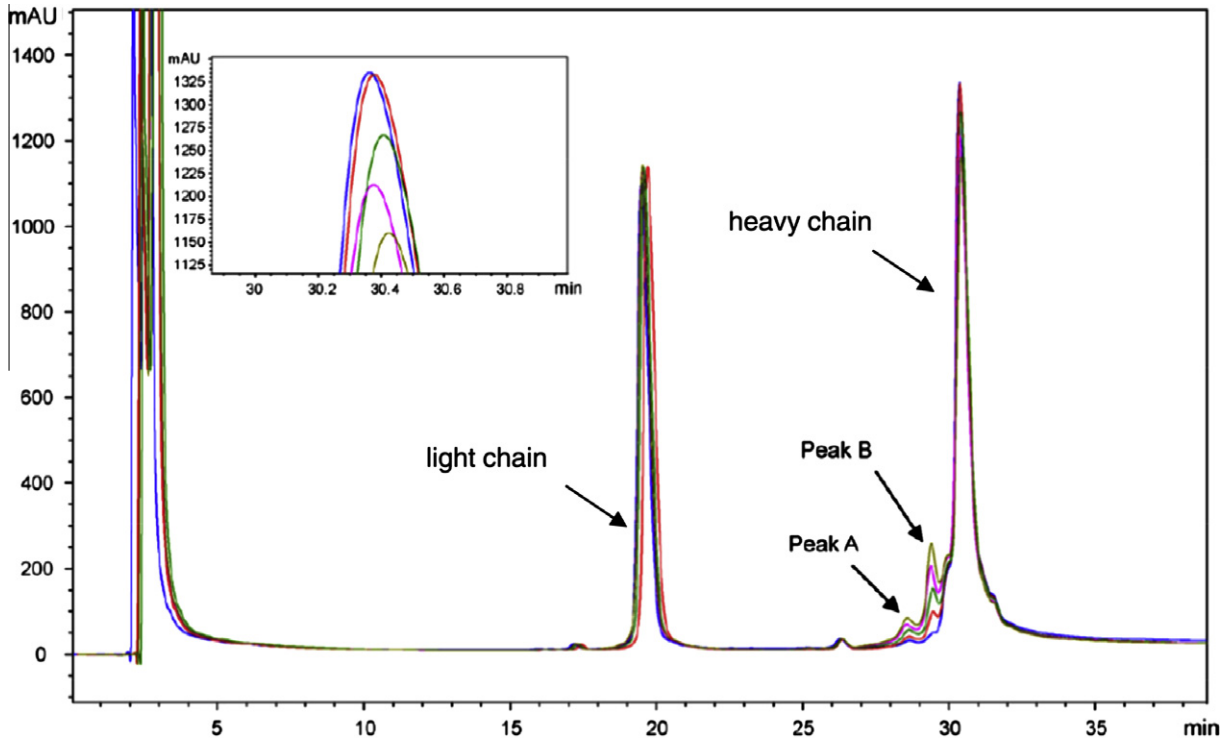


Figure 3. Reversed-phase liquid-chromatographic separation of oxidized heavy-chain variants (peak A and peak B) of a monoclonal antibody. The inset shows a zoom of the peak apex of overlaid HC peaks. Column: PLRP-S 1000 Å, 5 μm, 4.6 mm ID × 50 mm (Polymer Laboratories/Varian, Amherst, MA, USA). UV detection at 215 nm. Column temperature was maintained at 75 ± 2°C. A linear gradient elution was employed using mobile phases containing 0.1% trifluoroacetic acid in water and 0.1% trifluoroacetic acid in acetonitrile at an initial flow rate of 1 mL/min (from [21] with permission).

portion of the HC (Fd) of an IgG2 molecule released by papain cleavage under mild reducing conditions was presented by Yan et al. [22]. These domains were thoroughly characterized by RPLC-ESI-TOF/MS.

Ren et al. presented an RPLC method for the analysis of mAbs capable of separating and distinguishing between different site-specific single amino-acid modifications [23]. The authors reported the sequence-specific separation of methionine oxidation and reduced disulfide bonds in proteins as large as the HC and Fab domain of the mAbs.

Another research group applied RPLC as a starting point to investigate the glycosylation of intact and reduced mAbs [24]. The LCs of the mAbs exhibited a sharp primary peak, while the HC mass spectra reflected the glycosylation-heterogeneity profile of each IgG.

A recent study also reported the advantage of RPLC separation of the mAb sub-domains (LC, HC, Fab, and Fc) containing several specific modifications (e.g., pyroglutamic acid, deamidation, isomerization, and oxidation) [25]. Deconvoluted ESI mass spectra of these domains revealed the modification profiles of these variants with high accuracy and resolution. This method allows the routine RPLC-ESI-TOF/MS analysis of mAbs with minimal sample preparation and high throughput compared with mAb peptide mapping.

2.3.2. Recent trends in RPLC and RPLC-MS for mAb analysis. In RPLC, one of the primary causes of peak broadening with mAbs is the slow molecular diffusion of these compounds due to their large mass, which leads to a poor mass-transfer mechanism, so it is necessary to increase the kinetic efficiency of conventional RPLC separations.

Recent developments in RPLC [e.g., ultra-high-pressure LC (UHPLC), columns packed with wide-pore superficially porous particles, organic monolith columns, non-porous materials, and porous layer open tubular (PLOT) columns] allow dramatic increases in the efficiency and the resolution of protein separations, even with intact mAbs of ~150 kDa. The mobile phase temperature can play a key role in improving the efficiency of mAb separations. At elevated temperatures, the diffusion of large solutes is enhanced; the viscosity of the mobile phase decreases, and the protein conformations can be significantly altered. Generally, higher temperatures lead to better kinetic efficiency and recoveries. However, at elevated temperatures, the risk of possible thermal degradation becomes more serious.

Krull and Rathore recently highlighted the advantages of UHPLC over HPLC for mAb samples [27]. Two mAb samples were reduced and alkylated such that the LCs and HCs could be compared using an UHPLC-MS platform to identify differences between both products. The LCs were identical, but the HCs, in contrast, exhibited consistent mass shifts of approximately 32 Da for all protein glycoforms in the biosimilar compared with the innovator company's drug that it was intended to replicate. Mass shifts of this magnitude (multiples of 16 Da) are often indicative of oxidation.

Another recent article demonstrated that columns packed with the latest wide-pore superficially porous particles achieved considerably better resolution and recovery of an intact mAb than did conventional columns packed with wide-pore fully porous particles [28]. This work emphasized that a lower hydrophobicity in the thin porous shell can be advantageous in the separation of large hydrophobic proteins or mAbs.

Although the number of papers on this topic remains limited, several examples illustrate that UHPLC and core-shell technologies are promising approaches for the analysis of mAbs. The advantages of high pressures and core-shell column technology are obvious, so dedicated materials have become commercially available from several sources. This new class of wide-pore material dramatically improves the RPLC performance for mAb study and demonstrates the promising future of RPLC in antibody analysis [27]. The use of small-diameter particles, superficially porous or non-porous packings, and relatively high flow rates has led to reproducible mAb separations over shorter timeframes than those required by conventional HPLC, with excellent resolution, sharp peaks, high peak capacities, and elevated sample throughput [27].

3. Electrophoretic approaches

Three CE modes are commonly used in the analysis of mAbs:

- CGE, which is also known as CE-SDS;
- CIEF; and,
- CZE.

The first two techniques are the capillary counterparts of the traditional SDS-PAGE and IEF that are carried out in gels and present several drawbacks, including extended analysis time, low efficiency, limited reproducibility, and use of toxic reagents. As reviewed elsewhere, the capillary format enables automation of the experiments and provides better resolution. In contrast to the in-gel techniques, CE also has the potential to be coupled to MS to improve the identification of the compounds by the separation of co-migrating analytes with differing mass-to-charge ratios [29].

3.1. Capillary gel electrophoresis (CGE)

CGE is used to determine the apparent molecular weight of molecules. In the field of mAbs, CGE allows assessment of product-size heterogeneity, purity, and stability. Sample preparation consists of heating the sample in the presence of a high concentration of SDS, which denatures the secondary and tertiary structures without

affecting the disulfide bonds, thus resulting in uniformly charged proteins. The capillary is subsequently filled with a sieving matrix composed of polymers (e.g., SDS giving rise to the name CE-SDS), leading to a separation based solely on the hydrodynamic radius of the protein.

This method is well established for the analysis of mAbs, with reports dating from the early 1990s. In 2008, a publication from Rustandi et al. presented a range of parameters that can be studied by CGE {e.g., the quantification of non-glycosylated heavy chains (NGHCs) or antibody stability with respect to such conditions as temperature, light exposure, buffer pH, and formulation [30]}. Similar to LC approaches, CGE can take place under non-reduced or reduced conditions with the latter providing electropherograms in which HCs and LCs are well-separated (Fig. 4). Fragmentation of murine mAbs was studied without reduction, indicating that increased temperature and buffer pH led to higher degradation [31]. In another paper, the ratio of non-glycosylated antibodies was determined for therapeutic tocilizumab under reduced conditions, showing an NGHC content of approximately 1.2% [32]. Structural isoforms not caused by the production conditions were highlighted without sample reduction by CGE. While only one isoform was observed for IgG1, the IgG2 electropherogram presented two peaks that were identified by applying a redox potential as disulfide bond-related species [33].

A method for monitoring IgG2 disulfide heterogeneity was developed and tested for several IgG1, IgG2, and IgG4 samples and showed doublet peaks only for IgG2 [34]. Among the peculiar structural features, a non-reducible thioether bridge was detected between the LCs and HCs of human IgG1 under reducing conditions prior to the identification of the amino acids by peptide mapping with tandem MS [35].

Two quantitative methods were also developed and validated following the ICH guidelines for analysis under reduced and non-reduced conditions; the first, using LIF detection, showed a limit of detection (LOD) similar to that of the SDS-PAGE (~10 ng/mL) [36], while the second, utilizing UV detection, enabled the determination of impurities at 0.16% of the main peak [37]. Both methods concluded that using a buffer pH of approximately 6.5 decreased the mAb fragmentation under non-reduced conditions. This conclusion was also supported by another research group that developed a method for IgG1 with and without reduction and achieved a limit of quantification (LOQ) of 20 μg/mL with a relative LOD for impurities down to 0.1% [38].

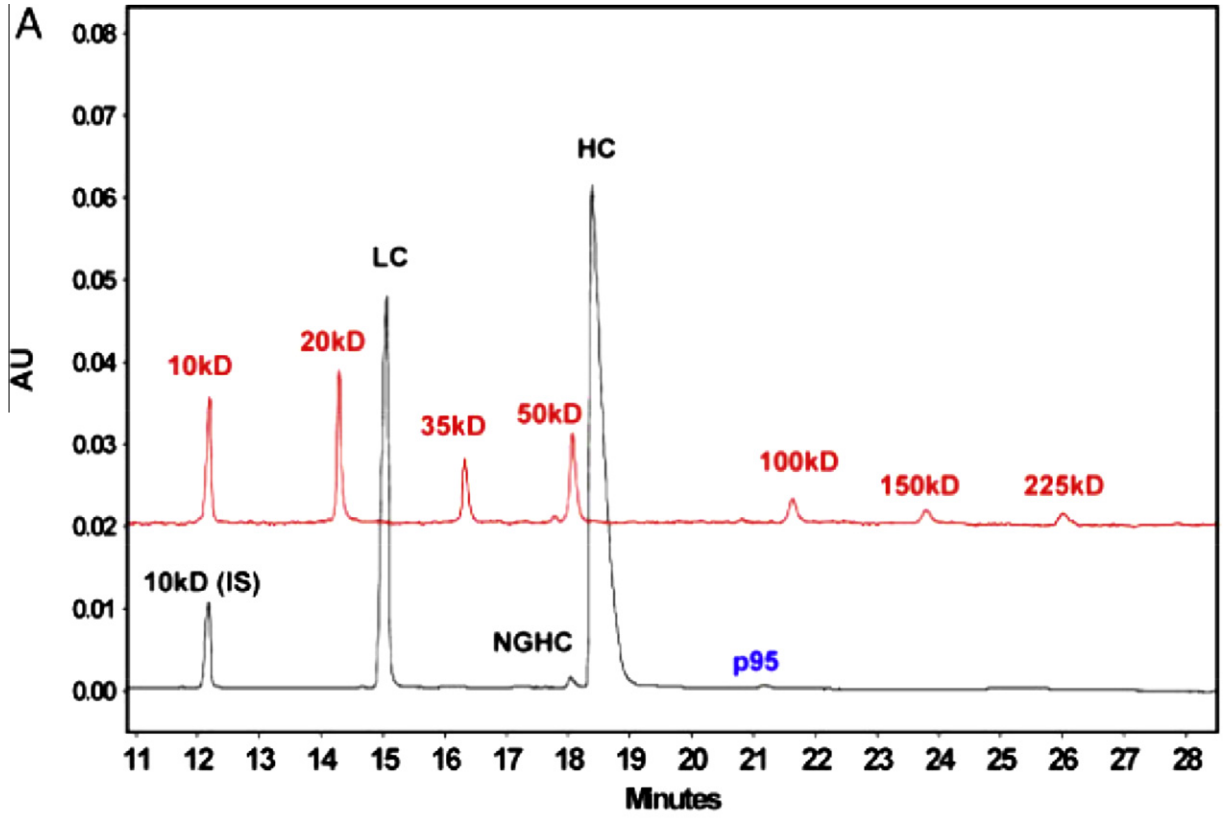


Figure 4. Capillary gel electrophoresis of a reduced mAb (lower trace) and molecular-weight markers (upper trace). HC, Heavy chain; LC, Light chain; NGHC, Non-glycosylated heavy chain; p95, mAb aggregated form (from [30] with permission).

3.2. Capillary-isoelectric focusing (CIEF)

Based on pI differences, CIEF is used to characterize the charge heterogeneity of biotechnology products that can be caused by degradation reactions. A pH gradient is formed inside the capillary, and the molecules of interest migrate under the electric field until their global charge becomes zero (the pH is equal to their pI). Three modes are commonly used in the capillary format:

(1) the conventional two-step method in which, in the absence of EOF, focusing is followed by a mobilization step to the detector;
(2) a one-step method involving a moderate EOF that mobilizes the analytes to the detector with simultaneous focusing; and,
(3) an imaging mode using a short transparent capillary with whole-column detection that obviates the mobilization step [39]. With this technique, the peaks obtained are generally classified into three groups: the main, the basic, and the acidic isoforms. Thus, a mAb is not only described by its pI but also by the content percentage of each isoform group.

The CIEF two-steps mode was used to evaluate the charge heterogeneity of gemtuzumab ozogamicin covalently bound to calicheamicin, an antitumor antibiotic [40]. The method was developed using a model IgG4 with a relative standard deviation (RSD) of 0.95% for the pI and lower than 5% for the percent composition of the charge. Other commercial mAbs were characterized in the same manner with an RSD lower than 0.5% for the pI determination of IgGR1 [41]. The same method was further applied to characterize C-terminal lysine variants and glycosylation profiles, demonstrating its value in clone-screening processes. Imaging CIEF (iCIEF or iCE) was compared with cationic exchange chromatography (CIEX) and exhibited similar peak profiles but shorter experimental times (20 min *versus* >60 min, respectively) [42]. The pI of the main species was determined with an RSD lower than 0.2%, and the proportions of the acidic, main, and basic species in the sample were quantified with RSDs of 1.9%, 0.9%, and 16.6%, respectively. Another publication reported the use of an identical approach applied to the determination and quantification of mAb-charge variants belonging to the IgG2 family [43]. More than 20 therapeutic mAbs with pI values in the range 6.9–9.6 and variable charge contents (10–70%) were evaluated with the method found in Fig. 5A.

3.3. Capillary-zone electrophoresis (CZE)

CZE is the most straightforward separation method among electro-driven separation techniques. The capillary is filled with a background electrolyte (BGE) and the separation proceeds according to differences in the analyte electrophoretic mobilities depending on their charge-to-size ratio. As previously mentioned for chromatography, one advantage of CZE, in contrast with CGE and CIEF, relies on its direct coupling with ESI-MS. CGE or CIEF combined with MS remains relatively complex,

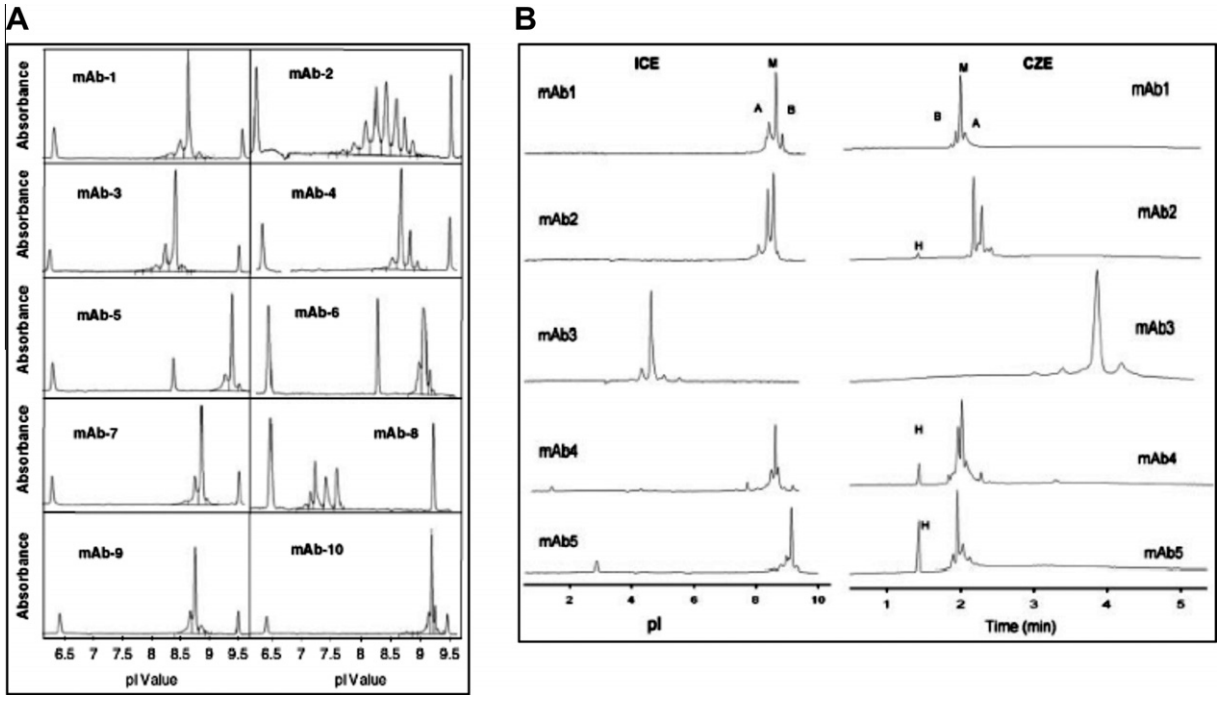


Figure 5. (A) Charge variant profiles of mAbs obtained by imaging CIEF (iCE) analysis (from [43] with permission). (B) Comparison of imaging CIEF (iCE) and CZE for the analysis of mAbs. M, Main peak; A, Acidic variant; B, Basic variant; H, Histidine (IS) (from [50] with permission).

due to the presence of non-volatile carrier ampholytes or polymers that hinder the ionization of mAbs. By contrast, CZE is very versatile in optimizing CE conditions affording easy compatibility with ESI-MS.

While several publications can be found on the analysis of intact proteins by CZE, mAbs remain relatively unstudied, probably due to their tendency to adsorb onto the negatively-charged surface of conventional fused-silica capillaries, so degrading their CE performance. However, this issue can be minimized by methods already in use for intact protein analysis (e.g., the use of extreme pH, high ionic strength, zwitterionic additives, organic solvents, and capillary coatings) [44], most of which circumvent the combination of CZE with MS.

In the field of mAb characterization, the CZE mode is primarily used to analyze glycosylation patterns by first enzymatically releasing the oligosaccharides from the protein backbone [45–47]. After derivatization with a fluorescent marker, glycans are separated by CZE and detected by laser-induced fluorescence (LIF) or ESI-MS coupled with LIF detection [46]. In these studies, apart from the glycosylation, the characterization of the intact mAbs was not performed by CZE.

Only three publications reported the use of CZE for mAb analysis. In 2009, an IgG1 was analyzed in a triple-layer-coated capillary, resulting in a reproducible, partially-resolved peak pattern [48]. One year later, He et al. developed a method capable of separating one IgG1 and two IgG2s using an uncoated capillary. This research also determined the percentage of acidic, main, and basic isoforms with an RSD lower than 8% [49]. By adding urea to the separation buffer, the method highlighted the presence of IgG2-disulfide isoforms. Finally, the method was modified using a dynamically-coated capillary and compared with an iCIEF method. The CZE technique achieved separation within 2–5 min., while 6–9 min. were required by iCIEF to obtain a profile similar to that shown in Fig. 5B [50]. The migration time and area percentage of the basic, main, and acidic isoforms were repeatable with RSD values of 0.14–0.19% for the migration times, and 2.4–7.3% for the peak areas. Unfortunately, BGEs and the additives employed in the aforementioned articles are incompatible with MS, but these studies pave the way for the CZE analysis of mAbs.

4. Conclusion

Separation techniques (e.g., chromatography and electrophoresis-based methods) are important tools for the characterization of therapeutic mAbs.

In chromatography, IEX has been widely employed for the determination of charge variants, while SEC is useful for the evaluation of aggregation or size variants. These ''historic'' techniques are widely used in the industry but suffer from limited resolving power (low kinetic performance) and an inability for complex coupling with powerful detection devices (e.g., ESI-MS). Many academic and industrial laboratories are therefore currently investigating the possibilities offered by RPLC for the analysis of therapeutic mAb samples. The analysis of mAbs in their intact form (~150 kDa) and the limited proteolysis approach, which involves producing some large fragments of 25 kDa, 50 kDa or 100 kDa, are the most promising. Thanks to recent advances in RPLC-column technology (e.g., the commercialization of fully porous sub-2 μm particles), core-shell particles, inorganic monoliths, and the availability of wide-pore stationary phases, the kinetic performance of these columns is excellent for intact and large fragments of mAbs, while the MS compatibility should be straightforward.

CE presents some obvious advantages over the classical gel procedure. CGE and CIEF approaches can be employed for the same purpose as SEC and IEX, respectively, and are used by the industry as reference techniques. Because of the significant developments and interest in ESI-MS over the last few years, and because CGE and CIEF are hardly compatible with ESI-MS, the CZE approach is being evaluated as a viable alternative that can be easily combined with ESI-MS.

Finally, one of the primary issues in RPLC and CZE relates to the adsorption of mAbs and their fragments. In RPLC, an elevated mobile-phase temperature of up to 70–90°C can dramatically improve the recoveries, while a suitable capillary-coating procedure can be employed in CZE to reduce the adsorption phenomena.

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