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Liquid phase separation techniques for the characterization of monoclonal antibodies and bioconjugates

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ABSTRACT

The characterization of biopharmaceuticals is a challenging task due to the structural complexity of such products. During both manufacturing and storage, biopharmaceuticals are exposed to a high risk of undergoing several desired and undesired chemical and enzymatic modifications. Final products are therefore heterogeneous mixtures of quite diverse chemical entities, rather than titrated blends of molecules. For this reason, the development of advanced separation techniques is paramount to allow the characterization of the different constituents of biopharmaceuticals. Several separation methods have been reported so far, each one based on specific physicochemical mechanism and uniquely suited for the characterization of distinctive product features, called Critical Quality Attributes (CQAs). If such diversity offers on one hand a wide array of possibilities, on the other hand it raises some issues concerning regulation, validation and harmonization of the results obtained in different laboratories or by different methods. Moreover, a comprehensive characterization of biopharmaceuticals can nowadays be achieved only by multiple analyzes with orthogonal methods or by the combination of orthogonal techniques in one method. Herein, we reviewed the main variants of Liquid Chromatography (LC), Capillary Electrophoresis (CE), and other less popular liquid phase separation techniques that are used for the characterization of monoclonal Antibodies (mAbs) and Antibody Drug Conjugates (ADCs).

1. Introduction

Biopharmaceuticals are becoming quite popular for the treatment of various diseases. Disregarding other products such as biosimilars or fusion proteins, in 2020 eleven antibody-based therapeutics were granted for the first approval both by US and European agencies (i.e., FDA and EMA), while 16 marketing application have been under revision in 2021 [1]. A detailed characterization of such products is important since any

Abbreviations: LC, Liquid Chromatography, RPLC, Reverse phase chromatography; CQAs, Critical quality attributes; SEC, Size exclusion chromatography; IEX, Ion exchange chromatography; HIC, Hydrophobic chromatography; HILIC, Hydrophilic liquid chromatography; UV, Ultraviolet-visible spectroscopy; FLD, Fluorescence spectroscopy; IR, Refractive index; LS, Light scattering; MALS, Multi angle light scattering; MALLS, Multi angle laser light scattering; IV, Viscometer; MS, Mass Spectrometry; MALDI, Matrix assisted laser desorption/ionization; TOF, Time of flight; ADC, Antibody drug conjugates; DAR, drug to antibody ratio; mAbs, Monoclonal antibodies; CEX, Cation exchange; AEX, Anion exchange; MES, 2-(N-morpholino) ethanesulfonic acid; CE, capillary electrophoresis; CZE, capillary zone electrophoresis; CIEF, capillary isoelectric focusing; QC, quality Control; AC, Affinity chromatography; LAC, Lectin affinity chromatography; ADCC, antibody cell-mediated cytotoxicity; PAT, process analytical technology; DoE, Design of experiment; QbD, Quality by design; DTT, Dithiothreitol; TCEP, Tris(2-carboxyethyl)phosphine; CCC, counter current chromatography; SMB, simulated moving beds; PCC, periodic counter current chromatography; SMCC, sequential multi-column chromatography; MCSGP, multi-column counter-current solvent gradient purification; GC, Gas chromatography; SFC, Supercritical fluid chromatography; BGE, Background electrolyte solution; EOF, Electroosmotic flow; MZE, Microfluidics zone electrophoresis; TDLFP, Transverse diffusion of laminar flow profile; HCP, Host cell proteins; LIF, Laser induced fluorescence; APTS, 8-Aminopyrene-1,3,6-trisulfonic-acid; 2-AA, 2-aminobenzoic acid, ESI, Electrospray ionization; SDS, Sodium dodecyl sulfate; CABCE, Carrier ampholytes-based capillary electrophoresis; LPA, Linear polyacrylamide; ITP, Isotachophoresis; IEF, Isoelectric focusing; FFE, Free-flow electrophoresis; iCIEF, Imaging capillary isoelectric focusing; CCD, Charge coupled device; CGE, Capillary gel electrophoresis; HPC, Hydroxypropyl cellulose; PEGDMA, Polyethylene glycol dimethacrylate; PVA, Polyvinyl alcohol; ANTS, 8-aminonaphthalene-1,3,6-trisulfonic acid; FQ, 3-(2-furoyl)-quinoline-2-carboxaldehyde; ACE, Affinity capillary electrophoresis; FTPFACE, Partial-filling affinity capillary electrophoresis; CEC, Capillary electrochromatography; BSA, Bovine serum albumin; PDDA, Poly-diallydimethylammonium chloride, MEKC, Micellar electrokinetic capillary chromatography; FDA, Food and drug administration; EMA, European medicines agency.

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small change in the structure of their constituents can lead to product instability, loss of activity or adverse effects like immunogenicity or other forms of toxicity [2]. However, the characterization of biopharmaceuticals is more challenging compared to other drugs, owing to the structural complexity of their constituents. Some of the structural features that uniquely distinguish monoclonal antibodies (mAbs) are their high molecular weight, a quaternary structure composed of light and heavy protein chains bridged by disulfide bonds, as well as the presence of glycoforms and other protein variants generated by a number of chemical or enzymatic modifications occurring upon protein expression [3]. Such features are not found in other drug classes.

Bioconjugates such as Antibody Drug Conjugates (ADCs) are other type of biopharmaceuticals accounting for more distinctive features such as the presence of payloads (i.e., small molecular drugs covalently linked to the antibody through an artificial chemical linker) and other structural modifications that are necessary for a specific and selective payload binding. Such modifications are product-dependent and are obtained by modification of the primary structure of antibodies by using chemical (i.e. disulfide reduction), enzymatic (e.g. via glycotransferases and transglutaminases) or biochemical processes (i.e. amino acid engineering)[4,5].

The huge variety of desired and undesired chemical and enzymatic modifications occurring during expression, purification, payload linking, or long-term storage, makes biopharmaceuticals more heterogeneous compared to other pharmaceuticals [6–8]. Regulation agencies have therefore introduced the concept of Critical Quality Attributes (CQAs) as specific product features that must be characterized[9,10]. Some CQAs are routinely monitored also for small injectable drugs like product appearance, pH, osmolality, particulates, leachable, bioburden, level of endotoxins, and sterility. Nevertheless, other CQAs have been introduced specifically for mAbs, namely the characterization of glycoforms, size variants, charge variants or oxidized protein species. Other additional CQAs are mandatory for approval of ADCs, like the characterization of drug-to-antibody ratio (DAR), distribution of payloads (DOP), along with the amounts of free drug, conjugation impurities and reagents or residual solvents[4,11]. However, CQAs are product-specific since the characterization of other features is mandatory for the approval of biosimilars[12,13], biobetter antibodies [14], glyco-engineered antibodies [15], fusion proteins and peptides[16,17], multi-specific antibodies [18] and antibody mixtures [19,20].

The characterization of CQAs requires the use of different techniques and analytical approaches specifically designed for the analysis of the structural peculiarities of each product. Since biopharmaceuticals are often heterogeneous mixtures of molecules covering a wide range of molecular weight and physicochemical properties, liquid chromatography (LC), capillary electrophoresis (CE) and other separation techniques have nowadays a key role in quality control of such products. Many different liquid phase separation variants have been reported so far, each one uniquely suited for the characterization of specific CQAs [11,21,22].

Both the complexity of biopharmaceuticals and the wide array of analytical approaches available, raised some regulatory issues concerning the harmonization and validation of the results [23]. The analytical procedures for the characterization of many CQAs with acceptance criteria for method validation have been published into ICH Q6B specifications. However, some CQAs must be determined around specific and peculiar features of either the products or the manufacturing processes, and cannot be applied universally to all biopharmaceuticals [24]. Another barrier for harmonization and validation of methods and protocols have been the availability of reference standards. Some attempts have been recently done with the development of mAbs standards (e.g., NISTmAb) [25], or standardized antibody derivatives resembling ADCs [26]. Such products have been successfully used for comparing different analytical methods [27], for interlaboratory result validations[28,29], and for biosimilarity assessments [30]. Although biopharmaceutical can be so diverse, some physicochemical properties are common across many products. Therefore, the implementation of the concept of Quality by Design (QbD) allowed the development of platform methods that are suitable for the analysis of multiple products. This should allow a faster and cheaper product development and a better harmonization of the analytical processes[31,32].

Some recent reviews have been published concerning the analysis of biopharmaceuticals with liquid phase separation techniques. However, such reviews are focused on specific products [11], on specific techniques [22], or they report only the main LC and CE variants with no mention of other less popular liquid phase separation techniques [3]. Therefore, we herein report a comprehensive and updated collection of the liquid phase separation methods available for the analysis of biopharmaceuticals, with a brief description of the main setup options for the characterization of specific CQAs.

2. Liquid phase separation techniques

The presence of specific impurities or structural features of the main constituent define the CQAs of biopharmaceuticals. The identity and quantity of principal components and impurities requires their separation before detection. For this reason, many variants of LC and CE are widely used for the characterization of biopharmaceuticals. Size Exclusion Chromatography (SEC), Ion Exchange Chromatography (IEX), and Hydrophobic Interaction Chromatography (HIC), Reversed Phase Liquid Chromatography (RPLC) and Hydrophilic Interaction Liquid Chromatography (HILIC) are the most popular LC variants reported in literature, while Capillary Zone Electrophoresis (CZE), Capillary Gel Electrophoresis (CZE) and Capillary Isoelectric Focusing (CIEF) are the most popular CE variants[3,11,22]. However, some authors reported also analytical applications for other liquid separations not classified as LC or CE variants, as well as for other less popular variants of both LC and CE[27,33–39].

Since the structure complexity of mAbs and ADCs, the characterization of their CQAs is typically performed at multiple structural levels. Specifically, biopharmaceuticals can be characterized maintaining intact their protein structure or after a sample preparation including a controlled sample degradation. Concerning intact protein analysis, it can be performed either in native condition (i.e., by preserving the conformation of protein constituents) or after denaturation (i.e., by disrupting the conformation of protein constituents). Denaturation is induced by organic solvents, detergents, pH jump or other variables influencing protein folding before or during the analyzes. Intact protein analysis is used to confirm the identity of mAbs by measuring the molecular weight, to identify the presence of size variants (e.g., antibody aggregates or fragments) or charge variants (e.g., antibodies having different isoelectric point), and to characterize other impurities. Concerning ADCs, intact protein analysis is also used to characterize Drug-to-Antibody ratio (DAR) and to measure the amount of free drug or other impurities such as conjugation reagents.

Controlled degradation of biopharmaceuticals can be instead used for Middle-Up or Bottom-Up experiments. In Middle-Up experiments, antibody domains are separately characterized after chemical degradation, proteolysis, or a combination of both. Disulfide reducing agents can be used to separate antibody heavy and light chains, while specific enzymes (e.g., IdeS, papain) are typically used to cleave mAbs and ADCs around the hinge region [40]. Middle-Up experiments are used to identify domain-specific charge variants and glycoforms, as well as to study the Distribution of Payloads (DOP) for ADCs.

Concerning Bottom-Up experiments, they are intended to characterize small molecules produced after enzyme degradation of biopharmaceuticals. Peptide mapping and the characterization of released glycans are the two most popular Bottom-Up analyzes. Peptide mapping is typically performed by using trypsin or other enzymes. This approach cleaves the proteins and produce a peptide mixture that is then separated and analyzed by tandem mass spectrometry to identify peptide

Table 1
Liquid phase separation techniques and their applications for the characterization of CQAs of mAbs and ADCs.

Technique	CQAs	Characterization level	Detectors
SEC	Antibody size variants	Intact	UV; FLD; RI; LS; MALS; MALLS; IV; MS
	Drug to Antibody ratio of ADCs	Intact	UV; MS
	Free Drug and small molecule impurities in ADCs	Intact (small molecule fraction)	UV; MS
HIC	Drug to Antibody ratio of ADCs	Intact	UV; MS
IEX	Antibody charge variants	Intact	MALS; UV; MS
AC	Antibody	Intact	UV; MS
	glycoforms	Middle-Up	UV; MS
	Antibody-	Intact	UV; MS
	dependent	Middle-Up	UV; MS
HILIC	Antibody	Middle-Up	UV; FLD; MS
	glycoforms	Bottom-Up	UV; FLD; MS
	Drug to Antibody ratio of ADCs	Middle-Up	UV; MS
	Distribution of Payloads of ADCs	Middle-Up	UV; MS
	Free Drug	Intact (small molecule fraction)	UV; MS
RPLC	Product	Bottom-Up	UV; MS
	identity/impurities	Middle-Up	UV; MS
	Antibody	Middle-Up	MALS
	size	Intact	MALS
	Drug to Antibody ratio of ADCs	Middle-Up	UV; MS
	Distribution of Payloads of ADCs	Middle-Up	UV; MS
	Free Drug and small molecule impurities in ADCs	Intact (small molecule fraction)	UV; MS
	Host Cell proteins	Bottom-Up	UV; MS
CCC	Antibody charge variants	Intact (preparative scale isolation)	UV; MS
CZE	Antibody	Intact	UV; LIF; MS
	charge	Middle-Up	MS
	Product	Intact	UV; LIF; MS
	identity/impurities	Bottom-Up	MS
	Host Cell proteins	Bottom-Up	MS
	Antibody	Bottom-Up	LIF; MS
	glycoforms	Middle-Up	MS
		Intact	MS
CIEF	Antibody	Intact	UV; LIF; MS
	charge	Middle-Up	UV
	variants	Intact	UV
CGE	Antibody size variants	Intact	UV; LIF
	Antibody	Bottom-Up	LIF; MS
	glycoforms	Middle-Up	UV
	Drug to Antibody ratio of ADCs	Intact	LIF
	Distribution of Payloads of ADCs	Middle-Up	LIF
	Product identity/impurities	Intact	UV; LIF
FFE	Antibody charge variants	Intact (preparative scale isolation)	MS
ACE	Antibody charge variants	Intact	UV
CEC	Antibody charge variants	Intact	UV
MECK	Antibody charge variants	Intact	UV
	Antibody glycoforms	Bottom-Up	LIF

sequences. Peptide sequencing allows the identification of the proteins originating the peptides and the localization of specific Post Translational Modifications (PTMs). Peptide mapping is therefore used to determine product identity (i.e., by mapping the antibody primary sequence), impurities such as Host Cell Proteins (HCPs) or antibody charge variants, glycoforms and oxidized species. Glycoforms are also characterized by Bottom-Up analyzes of glycans released from antibodies after treatment with specific enzymes (i.e., PNGase). Table 1 summarizes the application of the main liquid separation techniques discussed in this review, reporting the CQAs that can be characterized, the characterization level (Intact, Middle-Up or Bottom-Up), and the main detectors available.

2.1. Liquid chromatography (LC)

RPLC is the most popular separation technique for the characterization of pharmaceuticals, especially for quality control of small molecules. However, a variety of other LC variants are routinely used for the characterization of biopharmaceuticals at different level (see Fig. 1) [3].

LC variants have been developed to separate analytes based on different mechanisms. However, a common trait across all the variants is the use of a column where a stationary phase is immobilized and continuously permeated by an eluent (mobile phase). The physicochemical interactions established by the analyte with the stationary phase and the eluent determine how long it takes to elute each analyte. Once eluted, the analytes are typically detected by Mass Spectrometry (MS) or by spectroscopy detectors measuring either the absorption (UV) or the fluorescence (FLD). The mobile phases available for elution determine whether the method is denaturing or not. Non-denaturing separations are performed using aqueous, saline eluents that maintain protein folding (i.e., native conditions), whereas denaturing techniques relies on eluents containing organic solvents or chemicals that induce protein denaturation. Non-denaturing methods can be setup by using SEC, IEX, and HIC, while RPLC and HILIC require denaturing eluents [41]. These features make such techniques complementary for structural characterization of biopharmaceuticals, since some CQAs can be characterized only in native conditions, whereas others allow or require the denaturation of mAbs and ADCs during analysis.

The analysis of biopharmaceuticals by LC differs from small molecule analysis since the adsorption of the analytes on the hardware of the system can be problematic. Therefore, system priming to saturate all the possible interaction sites is often required. Priming is performed by injecting several times a protein such as bovine serum albumin (BSA), although such procedure might not cover all possible interaction sites [42]. Recently, several materials have been developed to overcome this issue. Titanium or polyether ether ketone (PEEK) have been successfully

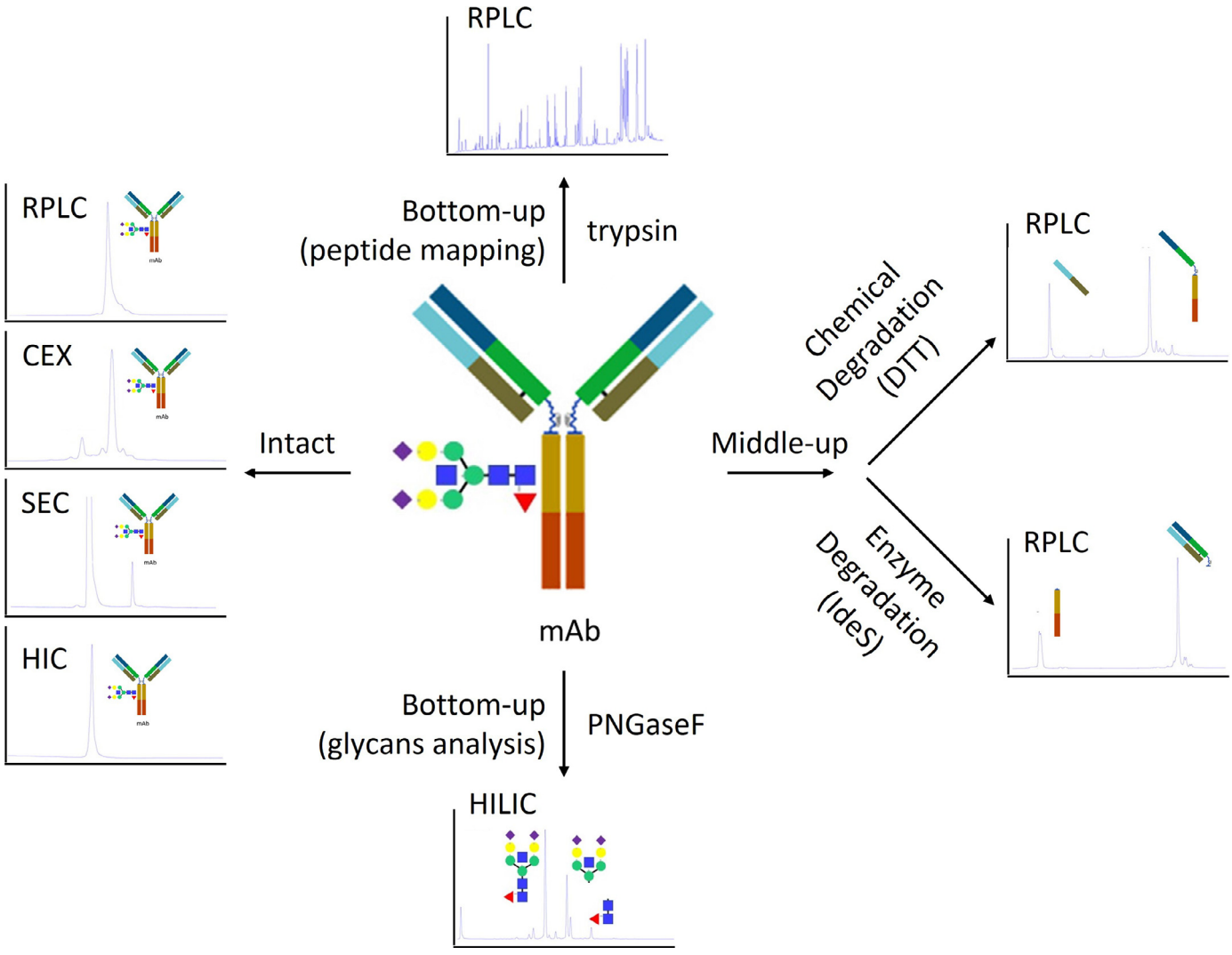

Fig. 1. LC approaches for the characterization of biopharmaceuticals.

tested. Compared to stainless steel columns, titanium support allowed to reduce the number of priming injections to reach a reproducible peak area of a standard mixture of proteins [42]. Titanium has also proven to be less prone to unspecific analyte adsorption and to be more resistant to corrosion [43], except when anhydrous methanol was used alone or with other solvents. However, corrosion can be prevented by adding a low percentage of water to the organic phase [44]. Metal columns coated with PEEK have also shown to minimize the unspecific adsorption of analytes on stainless steel surfaces. However, PEEK is hydrophobic and require a correct conditioning to avoid hydrophobic interaction with the analytes [45]. In fact, PEEK columns adsorbed more analytes, although less injections were required to remove adsorbed species during priming [42]. The downside of PEEK is its low mechanical stability to pressure and a poor reproducibility of inner diameter in PEEK column[42,45]. Moreover, PEEK frits were reported to be less permeable than the corresponsive metal counterparts, yielding to a higher backpressure in the same chromatographic conditions [42]. Other materials are currently under evaluation (e.g. MP35N, ceramic, and hybrid inorganic organic surfaces) to develop the best material for biopharmaceutical analysis [45]. If in one hand adsorption can be an issue during the analysis of mAbs, on the other hand it can be useful for purification. A recent paper reports the use of temperature-responsive columns, where electrostatic and hydrophobic interactions are used to provide mAbs adsorption at high temperature and elution of impurities. Antibodies can be then recovered by reducing the temperature and induce their elution [46]. The pore size of stationary phase is another critical feature during the analysis of biopharmaceuticals. A 160 Å pore size material can normally host analytes with a mass below 15 kDa, but not antibodies [47]. Materials with larger pore size (i.e., up to 1000 Å) have been then developed[48,49]. Moreover, superficial porous particles have been tested to overcome problems associated to the low diffusion coefficients of biopharmaceuticals. However, pore size seems to be the most critical feature for separation of biopharmaceuticals, while an increase of the particle size can be beneficial since it reduces the system back pressure [50].

Since different LC variants are based on orthogonal separation principles and suited for the analysis of different CQAs, multidimensional chromatography is an emerging strategy to collect a comprehensive characterization of mAbs and ADCs within a shorter analysis time [51]. Multidimensional chromatography can be applied in different ways. Comprehensive mode (LCxLC) are methods where all the eluents of the first- column are transferred into the second column. Conversely, in heart-cutting methods (LC-LC) only few selected fractions of the first separation are analyzed with a second column [21]. Since some LC variants are mostly incompatible with MS detectors (e.g., HIC; SEC, IEX), multidimensional methods are also used to bridge such techniques to MS via another technique that uses MS-friendly eluents (e.g., RPLC).

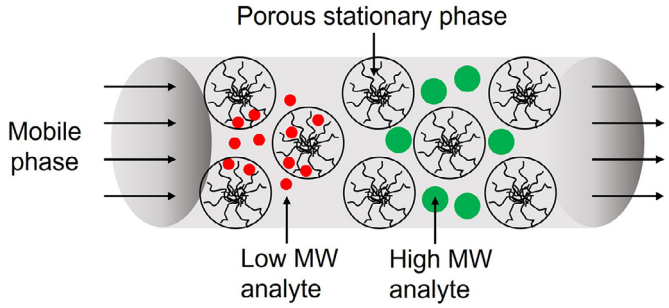

Fig. 2. Separation mechanism of a SEC column.

2.1.1. Size exclusion chromatography (SEC)

SEC is the state of art for the analysis of aggregates or fragments of biopharmaceuticals (i.e., size variants) by liquid chromatography [52]. SEC separations can be developed using eluents that preserve protein conformation. Size variants can be therefore characterized in native conditions. Separation is entropically driven, meaning that there is no retention of analytes on the stationary phase and elution is based solely on the different accessible column volume for analytes of different size [53]. The stationary phase is generally made of spherical silica beads with controlled pore size. High molecular weight analytes will elute first because they are not able to access most of the pores of the stationary phase. On the contrary, low molecular weight analytes elute later since they permeate more the stationary phase (see Fig. 2).

When SEC is used for separation of biopharmaceuticals, it must be kept in mind that protein conformers can elute at different time if their shapes (i.e., Rod-like, globular, or flexible chain structures) determine substantial differences of size [52]. The separation of conformers by means of SEC have been reported especially for aggregating proteins [54]. A recent paper, however, reported the separation of the conformers of a trispecific anti-HIV antibody in non-denaturing conditions [55]. SEC results were cross validated with other analytical technique and no evidence of species at different hydrodynamic radii have been found [56]. The elution of multiple peaks could be therefore due not to conformer separation, but to non-specific interactions with the stationary phase or hardware. In fact, such issues were not addressed during method development. On the contrary, another paper reports a SEC method for the separation of mAbs conformers developed to minimize unspecific interactions [57]. Specifically, a bidimensional SEC x SEC separation was developed to allow conformer separation on a first SEC column. Since the analytical conditions were not compatible with ion mobility-mass spectrometry (IMMS), a second SEC separation was used to allow the use of this type of detection. The authors therefore concluded that SEC could provide conformer separation since IMMS data and a parallel detection with Multi Angle light Scattering (MALS) revealed that species with different sizes, but same molecular weight were separated. However, species having smaller size were eluted earlier, which is in contradiction with SEC separation principles.These two examples demonstrate how non-specific interactions can negatively impact on SEC separations.

The two most common unwanted interactions in SEC are electrostatic and hydrophobic interactions. Concerning electrostatic interactions, when analytes and stationary phase have net charges of the same sign the repulsion forces decrease the elution time (i.e., ion-exclusion interactions). On the contrary, the elution is delayed when the analytes and the stationary phase have net charges of the opposite sign (ion-exchange interactions) [58]. Alternatively, an increase of the elution time can also be triggered by hydrophobic interactions between the analytes and the hardware. Such effect can be minimized by replacing stainless steel hardware with other materials such as titanium or PEEK [42]. Alternatively, the modification of the mobile phase is the most common strategy to minimize any unwanted interaction between the analytes and the stationary phase. Since SEC eluents are mostly aqueous solution, ionic strength and pH play an important role for performances. Increasing the ionic strength can reduce interactions of charged analytes[58,59]. For biopharmaceuticals, the optimal pH of mobile phase is at protein isoelectric point (pI), since the net charge of the analytes is neutral and chances of ionic interaction are therefore minimized. However, to avoid protein denaturation with aggregation or precipitation, a nearly neutral pH is required for the analysis of many biopharmaceuticals. Since silica is the most common material for SEC, stationary phase exposes acidic groups (i.e., sylanols) that are negatively charged at nearly neutral pH. Therefore, ion-exchange is triggered by eluents at pH below the pI of the analytes, while ion-exclusion is induced by eluents at pH above the pI of the analytes. The use of basic ionic additives (e.g., arginine) can therefore improve the separation by reducing secondary ionic interactions [59]. Organic solvent (i.e., methanol) can also be added at lower percentage (5–10%) to break hydrophobic interactions of proteins with stationary phase, without denaturation [52]. However, some SEC additives are not optimal for the coupling with some detectors or for native conditions that are necessary for conformer analysis.

Another critical parameter is temperature, which can be increased to reduce solvent viscosity and to enhance the analyte diffusivity and mass transfer. This can reduce the elution time and increase the resolution [60]. However, a high temperature can also trigger protein denaturation. If in one hand denatured proteins can elute faster since the unfolding is generally associated to an increased apparent size, extreme denaturation can lead to protein aggregation and precipitation.

SEC can be hyphenated to several detectors like the already mentioned UV, FLD, MALS and MS, although also refractive index (RI), light scattering (LS), multi angle laser light scattering (MALLS) or viscometer (IV) have been reported[52, 59]. Among all these possibilities, the most popular chaice for biopharmaceuticals is UV. Absorbance of aromatic amino acids of mAbs and ADCs is used for the detection at 280 nm. Aromatic amino acids (especially tryptophan) allow also fluorescence detection, with a typical excitation wavelength at 280–295 nm and emission at 300–370 nm [61]. Compared to UV, fluorescence has a higher sensitivity, allowing the identification of smaller quantity of contaminants. However, when SEC is used to analyze size variants, it must be kept in mind that protein aggregates typically have a higher intrinsic fluorescence. MS can also be directly connected to SEC by using non-denaturing solvents allowing to perform native protein analysis [59]. Compared to other detectors, MS has a small list of compatible eluent additives, since only volatile salts such as ammonium acetate, ammonium formate, and ammonium bicarbonate can be used [52]. Besides salt type, concentration may be critical as well, and the suitable combinations of salt type, pH and ionic strength should be carefully evaluated for each protein to avoid analyte denaturation, electrostatic and hydrophobic interactions but also ionization suppression during MS detection [62]. Since the direct coupling of SEC and MS is tricky and sometimes impossible, several problem-solving strategies have been developed. One method involves the use of off-line mass detector such as MALDI-TOF instruments [63]. Recently, metal free hardware has been also developed to allow direct SEC-MS hyphenation, without metal interferents [64]. Another alternative is to connect SEC column to a RPLC column. This create a two-dimensional chromatography, enabling the replacement of SEC additives not compatible with MS [65].

As already mentioned, SEC is mainly used for the characterization of size variants (aggregates and/or fragments) of mAbs [66] and ADCs [67], since such species have a different size compared to intact antibody monomers. SEC is used also in multidimensional methods to isolate size variants that are then characterized by Bottom-Up approaches [68], or to characterize aggregates of mAbs in harvested cell culture after a first purification step by affinity chromatography [69]. Concerning the characterization of ADCs, SEC is applied also for the determination of other important CQAs. Specifically, SEC-MS is used to measure drug to antibody ratio (DAR)[67,70,71], while multidimensional chromatography

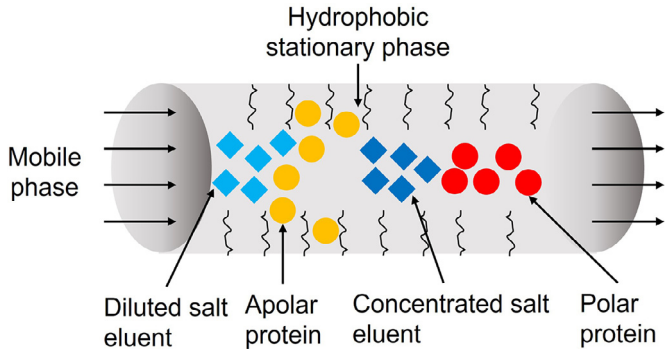

Fig. 3. Separation mechanism of a HIC column.

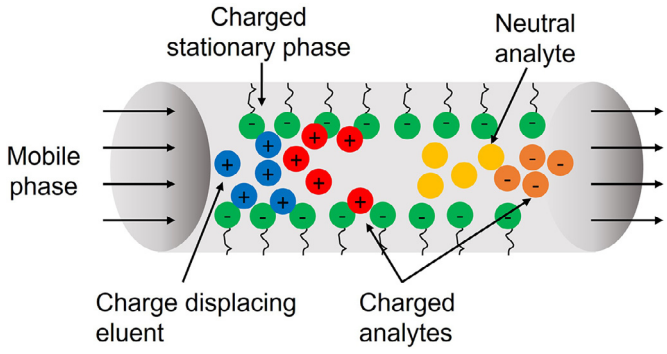

Fig. 4. Separation mechanism of a CEX column.

with SEC at first dimension was reported for the analysis of free drug [65]. Similarly, small molecular weight impurities of formulated mAbs can also be easily separated from antibodies by using SEC. For instance, trans-urocanic acid and other impurities have been characterized by a multidimensional method using SEC as first dimension [72].

2.1.2. Hydrophobic interaction chromatography (HIC)

HIC is a non-denaturing separation that is widely used for biopharmaceuticals analysis. Some typical applications of HIC are the monitoring of post-translational modifications, the characterization of the hydrophobicity and related impurity of mAbs and ADCs, and the determination of drug-to-antibody ratio of ADCs [73]. HIC separation relies the so-called salting out effect, which depends on the hydrophilic/hydrophobic balance of proteins[73,74]. Briefly, in highly concentrated saline solutions, water molecules cannot efficiently solvate both proteins and salts. When protein hydrophobic interactions prevail over water solvation, protein aggregation and precipitation typically occur. The more the proteins are hydrophobic, the lower is the salt concentration inducing protein aggregation and loss of solubility. Into a HIC column, however, insufficiently solvated proteins can interact with the lipophilic stationary phase. For this reason, HIC columns retain proteins when they are permeated by highly concentrated saline solutions. Separation according to protein hydrophobicity can be then achieved by reducing salt concentration over time (reverse salt gradient, see Fig. 3)

In fact, when the saline concentration of the eluent is gradually reduced the proteins become more soluble in the mobile phase and less retained by the stationary phase. The more the proteins are hydrophobic, the higher is their retention time because elution require a lower saline concentration into the mobile phase.

HIC stationary phase is made of silica or other polymers derivatized with n-alkyls, phenyl or ether moieties providing the desired hydrophobicity, which is not as high as for RPLC[75,76]. Several parameters can have a huge impact on the performance of the chromatographic separation. Salt type and concentration are very important. Concerning anions, phosphate, acetate, chlorine, and sulfate are typically used, while ammonium is the most popular cation [76]. However, the same salt can have different performances on different stationary phases, so the choice of the salt is usually done during the first phases of method development [77]. Although the typical salt concentration at the beginning of the gradient ranges from 1 to 5 M, the replacement of salts can require gradient adjustments [73].

The pH of the mobile phase can also impact on the interaction of the stationary phase with analytes such as mAbs or ADCs. As already mentioned, analyte net charge is influenced by protein pI and by the pH of the eluent. Protein solubility and overall hydrophobicity depends also on net charge, and eluent pH can therefore modify the interaction with the stationary phase. Extreme pH values of the eluent can induce denaturation, so HIC can be used also for protein separation in denaturing conditions. Nevertheless, HIC is mainly useful to study biopharmaceuticals in native conditions [73]. Organic modifier (e.g., water-miscible alcohols) can also be added to the system. Isopropanol is often added to reduce the strength of the interaction of the protein with the stationary phase and reduce run time (https://jcsmr.anu.edu.au/files/hic_handbook.pdf). As for pH modification, also organic modifiers can induce protein denaturation so they are usually added at low percentage (typically ≤ 15%). In HIC the reverse salt gradient is usually provided by mixing at different proportions concentrated and diluted saline solutions. Organic modifiers are typically added to the diluted saline solution to avoid protein precipitation when the saline concentration of the eluent is high. When this setup is used, an increase of organic modifier occurs over time along with the decrease of salt concentration [73].

Concerning the characterization of CQAs, HIC is the gold standard for the determination of drug-to-antibody ratio of ADCs. In fact, conjugation typically increases the hydrophobicity of antibodies and allows the chromatographic separation of the species according to the number of linked payloads [78]. UV is the most popular detector, while the elevated concentration of non-volatile salts required for elution makes HIC mostly incompatible with MS [79]. However, offline or online 2D chromatography using RPLC as second dimension and HIC as first dimension were developed to overcome the HIC-MS compatibility issue[80,81]. Recently, direct HIC-MS hyphenation for native condition analysis has also been reported. To provide good nebulization even at high concentration of salts, volatile electrolytes like ammonium acetate were used instead of the most common non-volatile salts. Moreover, new interfaces between HIC and MS were tested to include post-columns flow splitting and mixing with a sheath liquid that enhances nebulization and ionization of the analytes[82,83]. Such platforms were used to characterize variants of mAbs generated from small post translational modifications (e.g., oxidation, different levels of glycosylation) showing a different hydrophobicity compared to the unmodified mAbs.

2.1.3. Ion exchange chromatography (IEX)

Ion exchange chromatography is the state of art for the determination of charge variants of therapeutic proteins [84]. IEX variants are grouped as cation exchange chromatography (CEX) if the stationary phase is negatively charged and retains cations. On the contrary the stationary phase of anion exchange chromatography (AEX) is positively charged and retains anions. In all IEX methods, the elution is achieved either by increasing the concentration of salts or by changing pH of the eluent. These variants are called salt-gradient based separations, and pH-gradient based separations (also known as chromatofocusing), respectively. In salt gradient based separations the increase of salt concentration elutes the analytes by disrupting their interactions with the stationary phase (see Fig. 4).

Similarly, in pH-gradient based separations the elution is provided by changing the eluent pH to neutralize the analyte net charge responsible for the interaction with the stationary phase. For salt-gradient based separation the pH of the mobile phase must be buffered in between the pI of the analyte and the pK_a of the functional groups on the surface of

the stationary phase [84]. In fact, to provide analyte retention, the eluent pH must keep the protein and stationary phase at opposite net charge values. Although the pH is buffered, small variations within the above-mentioned range have an impact on the strength of the interaction of the protein and separation performances. Typically, the eluent pH ranges from 5.5 to 7.0, while the most popular compounds used to buffer the eluents are salts of phosphate (pK_a = 7.2), citrate (pK_a = 3.1), 2-(N-morpholino) ethanesulfonic (MES, pK_a = 6.1) or 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, pK_{a1} = 3.0, pK_{a2} = 7.5). Buffer concentration is typically 10 to 50 mM, while salt gradients are usually provided by NaCl or KCl up to 1 M. For pH-gradient based separation, it is very important to cover a wide pH range during elution[85,86]. This is typically achieved by mixing weak bases and acids at different proportions. Piperazine, Tris(hydroxymethyl)aminomethane (Tris), triethylamine, and ammonium hydroxide are some common bases used to change eluent pH[87,88]. A gradient form acidic pH to basic pH is used for CEX columns, while a gradient from basic to acidic pH for AEX columns. In chromatofocusing, elution occurs when net charge is neutral. For protein this require that eluent pH is equal to protein pI. However, gradient and pH range can vary with the analytes since each protein has a different pI that is a crucial attribute for this kind of application [84].

Stationary phases are divided in two categories: strong-exchangers and weak exchangers [84]. Strong exchangers stay ionized for a wide pH range because they have strong acidic or basic moieties. This allows a stronger interaction with proteins and might require the use of high concentration of salts or extreme pH values to provide elution. On the contrary, weak exchangers have strong acidic or basic moieties and elution can be provided with lower concentration of salts or smaller variation of the eluent pH, compared to strong exchangers. The stationary phase and elution condition (pH-gradient or salt-gradient based separation) must be chosen carefully based on protein features (i.e., liable proteins might require elution in mild conditions) [89]. As for other LC variants, analyte adsorption is critical when biopharmaceuticals are analyzed by IEX. Anomalous adsorption of mAbs can be observed as an effect of the asymmetric charge distribution on the surface of the molecules, therefore hardware and eluents must be carefully developed [90]. For weak cation exchangers, titanium or PEEK coating have superior performances compared to the stainless steel counterpart in the recovery and resolution of the analytes [42]. Recently, new type of short columns with non-porous solid phase material were developed, tested and good resolution was obtained in UHPLC system even at one minute run time [91].

IEX is mainly used for the characterization of charge variants of mAbs and ADCs. Besides IEX, capillary electrophoresis (i.e., CZE and CIEF) can be used as well. Although CIEF has been proven to be the gold standard for this analysis [92], IEX is quite popular in QC labs. CIEF outperforms IEX especially since structural modifications of mAbs have a more critical impact on IEX performances [93]. However, IEX can be successfully used in multidimensional separations to characterize DOP of ADCs, especially when lysine is used as liking site for payloads [94]. Multidimensional separations based on IEX can also be used for the characterization of charge variants by using Middle-Up or Bottom-Up approaches. For example, a combination of CEX with RPLC have been reported for the characterization of charge variants both at intact protein level and Middle-Up level [95]. Concerning Bottom-Up analyzes, a 4D-LC-MS method have been developed for the separation of charge variants by IEX, followed by online enzymatic digestion and peptide analysis[96,97]. Besides analytical purposes, IEX can also be used to purify mAbs. Specifically, flow through IEX can be used to elute antibodies while retaining process/product-related impurities and exogenous/endogenous viruses [90].

UV has been widely used as detector for IEX separations, although recently MALS has been reported too [98]. Although IEX has in important role in the characterization of biopharmaceuticals, the use of high concentration of salts limits its hyphenation with MS. As already mentioned

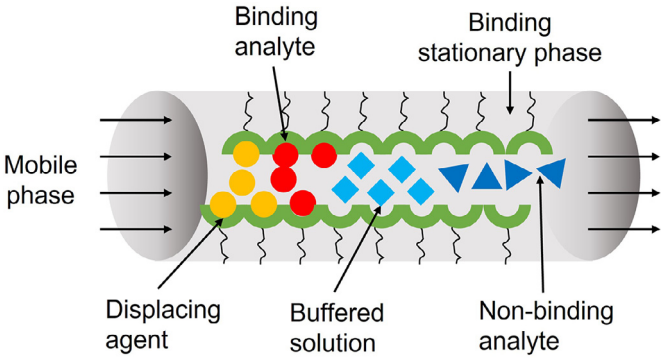

Fig. 5. Separation mechanism of an AC column.

for SEC, 2D systems with a RPLC chromatography as the second dimension can be employed to provide MS detection of species eluted from IEX separations [94]. Moreover, as for HIC and SEC, new applications for the direct hyphenation of IEX chromatography for native analysis by MS have been published. Such methods are used for the detailed characterization of antibody charge variants [99–101]. To allow the direct hyphenation, flow splitting and replacement of eluent salts with volatile additives were the main strategies. As already reported for HIC and SEC, ammonium acetate resulted the best salt for providing good separation performances, simultaneously allowing MS detection.

2.1.4. Affinity chromatography (AC)

Affinity Chromatography can be used for both sample preparation and analytical purposes. Retention is achieved exploiting biologicals interactions between the analytes and the stationary phase. Antibody-antigen, enzyme-substrate, and metal-phosphorylated-analytes are some examples of such diverse and complex mechanisms [102]. One of the two species providing such interactions (i.e. affinity ligand) is immobilized on an inert chromatographic support and packed in a column [103]. Sample loading is achieved by injecting the analyte dissolved into a solution that must not contain interferents that break the interactions between analytes and the affinity ligand. After sample loading, the system is washed to eliminate impurities and non-specific binders that might be adsorbed on the stationary phase. Elution is then achieved by eluents that break the interactions of the analytes with the affinity ligand, owing to a pH jump, a change of ionic strength or the presence of compounds competing with the analytes for affinity ligand binding (see Fig. 5) [103].

Antibodies, Ig-binding proteins (e.g., Protein A), Fc-Receptors, boronate, lectin, and immobilized metals are the most popular affinity ligands [102], while the list of reagents that can be included in the eluent is very limited compared to other chromatographic separations. Organic solvents such as acetonitrile can't be employed since they induce denaturation of the affinity ligand, especially in case of proteins. Therefore, loading is mostly done with aqueous buffer within a pH range of 3 to 8. Loading buffer has the role of activating the affinity ligand enabling its interaction with the analytes [103]. Temperature has also a critical role since it can induce denaturation of thermo-liable affinity ligands[103,104].

Affinity chromatography has a central role in the separation and characterization of antibody glycoforms. Glycation of monoclonal antibody is considered an important CQA since can influence the activity, the stability, and the quality of the products. Since the production of these biopharmaceuticals is made in living system, glycosylation patterns might change between batches and its characterization should be carefully addressed [104]. The characterization of glycoforms can be performed both in native conditions and by Middle-Up experiments. The most popular affinity ligands for such analyzes are lectin, boronate, and FcγRIIIa.

Lectin Affinity Chromatography (LAC) exploit the ability of lectins to interact with glycans. Selectivity on different sugar is obtained by using different lectins, since they have affinity to specific sugars. The binding between lectin and the corresponding sugar is noncovalent, specific, and reversible. Elution is carried out via competitive elution using specific sugars as eluent additives [105].

Boronate Affinity chromatography (BAC) involves the interaction of the tetrahedral anion of boronic acid with cis-1,2-diols, which are common moieties of sugars. Therefore, boronate chromatography can be used for the separation of glycoforms of therapeutic proteins. The interaction between the analytes and the stationary phase is carried out at alkaline pH and can be used only on proteins that are not poorly soluble in such conditions (e.g., protein with high pI). Elution is carried out either by lowering the pH or by competition of low molecular weight polyols such as sorbitol or Tris. To reduce nonspecific interactions between non glycosylated proteins and the stationary phase, small amounts of poly hydroxyl-chemicals can be added during sample loading to avoid nonspecific binding (i.e., shielding boronate chromatography). Concentration is then increased to provide binding competition and elution of specifically-bound analytes [106].

Another AC for the characterization of glycan variants is the chromatography that uses the Fc Receptor ɣ IIIa (FcɣRIIIa) as affinity ligand[104,107,108]. IgG1 subclass is the main interactor, along with IgG3 and IgG4. On the contrary, IgG2, IgA, and IgM don't bind FcɣRIIIa [104]. This AC variant is selective for N-glycated forms located at the highly conserved Asn-297 residue in the Fc region of mAbs. Such N-glycans are well-defined structures with a biantennary shape composed of a heptasaccharide core [104]. Critical sugar moieties that can drastically influence binding to the receptor are galactose and fucose[104,109]. The presence of the glycosylation on the Fc of antibodies is mandatory for the interaction with the receptor, and *in vivo* the activation of the receptor yields to the so-called antibody dependent cell-mediated cytotoxicity (ADCC). In fact, there is a correlation between the binding strength in FcɣRIIIa-based AC and ADCC *in vivo*. ADCC is one of the CQAs that must be usually evaluated for biopharmaceuticals, especially for mAbs and ADCs.

Since AC columns are intended for multiple analyzes, it is important to provide elution without affecting the structure and therefore the activity of the affinity ligands. For this reason, column conditioning before sample loading is very important. Affinity chromatography can be easily hyphenated to UV detector or MS. UV detection is more straightforward, while MS detection requires the use of volatile salts during elution, to avoid suppression during the ionization phase [104].

Affinity chromatography has also a central role in antibody purification both at preparative scale for manufacturing processes and at analytical scale for sample preparation in quality control. FcɣRIIIa AC, boronate chromatography, and lectin affinity chromatography can also be used for the purification and isolation of glycoforms, before further analyzes aimed at their characterization. However, Protein-A-based columns are the most popular support for purification by affinity chromatography. Protein-A was firstly discovered on the cell barrier of *staphylococcus aureus* and it binds the heavy chain within the Fc region of most immunoglobulins and within the Fab region of some antibody classes [110]. It is used as first step in downstream processes of antibody production, to separate the antibody from the cell culture broth. Upon purification with Protein-A, other LC variants can be used to characterize CQAs of the products. A bidimensional Protein-AxSEC method was reported for the characterization of titer and aggregation of mAbs in the harvested cells [69]. Protein-A purification can be also employed before Bottom-Up analysis of products both at peptide level [111] or by analyzing released glycans [112]. The use of an array of other separation techniques such as SEC, CEX, and RPLC-MS was reported to be useful to comprehensively characterize the products and allow clone selection for biosimilar production [113]. Finally, a MS-hyphenated Protein-A method was developed for the native analysis of mAbs in upstream processes, with the goal of implementing it as process analytical technology (PAT). The setup consist of a rapid sample cleanup, followed by elution and MS analysis of mAbs, including system re-equilibration and column conditioning before the next analysis [114]. Besides Protein-A, other specific immunoglobulins binders exist like Protein-G from streptococcal bacteria [115] and Protein-L from *Peptostreptococcus magnus*I [116]. Like Protein-A, also protein-G binds the Fc region or the Fab region of immunoglobulins, while Protein-L specifically binds antibody light chains.

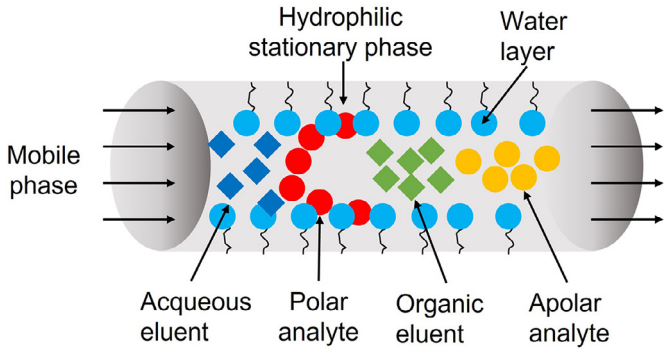

Fig. 6. Separation mechanism of a HILIC column.

2.1.5. *Hydrophilic interaction liquid chromatography (HILIC)*

HILIC is a chromatography variant developed for the retention and separation of hydrophilic compounds. The stationary phase is made with derivatized silica or polymers and can expose multiple kind of moieties, from polyols to ionizable group such as carboxylic acids or amides. The separation is based on analyte partitioning between a hydrophobic eluent and a hydrophilic water-enriched layer covering the stationary phase. The sample loading is provided by a mobile phase composed of water containing a high percentage of water-soluble organic solvents (e.g., acetonitrile), whereas analyte elution is achieved by increasing the water percentage (see Fig. 6) [117].

Eluent pH and ionic strength affects the separation by establishing a competition between the mobile phase and the analytes for creating an interaction with the stationary phase. Salt type and eluent pH must be determined based on the characteristics of the analytes and the stationary phase. As already reported for other chromatography variants, volatile salts must be used If HILIC is coupled to MS. Ammonium formate and acetate are the most popular salt reported for direct HILIC-MS hyphenation [117].

As for other chromatography variants, mAbs and ADCs suffer of adsorption processes on the HILIC stationary phases and a high operating temperature was reported to reduce adsorption [118]. In detail, adsorption of mAbs was reported to be less pronounced already at 60 °C, whereas higher temperatures (i.e., 80–90 °C) can further minimize or eliminate such an issue. Concerning the analysis of ADCs, adsorption was more evident for payload-linked species, and only an eluent temperature above 80 °C was sufficient to have a good recovery of all analytes to determine the product DAR. Like other LC variants, also HILIC requires a wide pore stationary phase for the analysis of large molecules such as biopharmaceuticals. If pores are not large enough to accommodate the analytes, size exclusion mechanisms can have a bigger impact than the interaction with the stationary phase. Although this have been proven for RPLC[48,119], only few studies with wide pore HILIC stationary phase are available so far [120–122]. New wide pore HILIC stationary phases should be therefore developed and tested. Column conditioning is also very important for HILIC, especially when it is used for the analysis of intact proteins. Compared to other LC variants, HILIC typically needs longer time to reach a good column equilibration and give reproducible separations. According to some studies equilibration can take up to one hour in isocratic mode[49,123]. A strategy to allow a better equilibration of the column during antibody analysis can

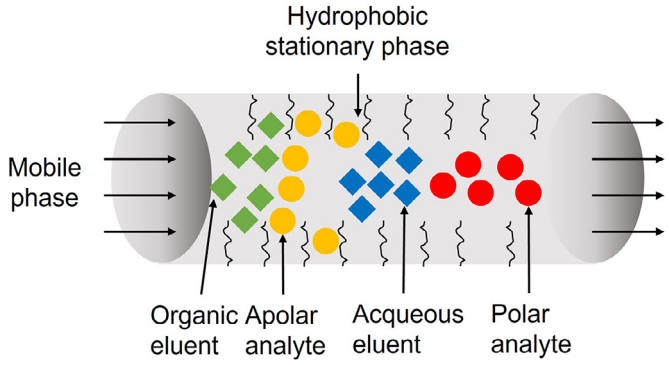

Fig. 7. Separation mechanism of a RP column.

rely on column conditioning through multiple injections of a standard. However, eluent additives such as trifluoroacetic acid (TFA) seems to allow a good analyte recovery and reproducibility even without such a conditioning step [124]. The same studies reported that storage solvent is also important, while superficially porous and totally porous stationary phases seem to perform similarly for biopharmaceuticals. Finally it was reported that pore size and temperature are also very important, although different materials and chemistry of the stationary phase can perform differently an require different adjustments during method development.

In biopharmaceutical analysis HILIC is used for its ability to retain and separate hydrophilic molecules. Specifically, HILIC is widely used for the characterization of antibody glycoforms. This is achieved at intact protein level [124], by enzyme degradation of mAbs and ADCs followed by the analysis of released glycans [125], or by Middle-Up experiments aimed at characterizing glycosylated substructures of mAbs and ADCs (e.g., glycosylated heavy chains or glycosylated Fc fragments) [120].

Since HILIC uses a high percentage of organic solvents during the run, it is easily compatible with MS detectors. Other detectors coupled with HILIC chromatography are FLD and UV[125,126]. Concerning the analysis of released glycans, detection is tricky since such analytes neither provides response to UV or FLD detectors, nor have ionizable groups allowing ionization and MS detection. Derivatization with fluorescent probes such as 2-aminobenzamide is therefore the most popular choice since fluorescence detection provides high sensitivity [125]. However, since most of the fluorescent probes have also ionizable groups (e.g., 2-aminobenzamide), MS detection upon derivatization is also a popular choice since it has the advantage of giving more structural information than FLD. When HILIC is used for the analysis of glycoforms at intact protein level, the use of TFA was reported to be compatible with MS detection, although TFA is normally avoided for MS analysis due to potential ionization suppression [124].

2.1.6. Reversed phase chromatography (RPLC)

RPLC is the most popular chromatography variant, and it is used for multiple purposes thanks to its robustness, selectivity, versatility, and compatibility with many detectors, including MS. Stationary phase is silica or polymer-based and is derivatized with n-alkane of different length (typically $n = 4$, 8 or 18). Short chain alkanes are used for Middle-Up or intact protein analysis, whereas C18 columns are normally used for Bottom-Up approaches. Retention mechanism is based on analyte partitioning between the hydrophobic stationary phase and a more hydrophilic mobile phase. Elution is provided by decreasing eluent polarity over time, through an increase of the percentage of water-soluble organic solvents (i.e., acetonitrile, methanol) in the eluent (see Fig. 7).

The use of organic solvents makes RPLC not applicable for native analyzes of biopharmaceuticals. However, RPLC is the first denaturing separation that has been implemented for the analysis of biopharmaceuticals, while HILIC has been introduced just recently. As for other chromatography variants, a big issue is the adsorption of mAbs, antibody fragments or ADCs on the stationary phase . Therefore, several strategies have been tested to minimize these processes through the modification of temperature, eluent compositon, or the system hardware, including stationary phase. Working at high column temperature (i.e., above 75 °C) is often necessary to have a good mass recovery for analytes like mAbs [127]. Analyte adsorption is more pronounced for ADCs, especially for species linked to many payloads. However, a high temperature could induce degradation or aggregation of liable analytes and must be carefully evaluated during method development [118].

Concerning eluents, water and acetonitrile are the most popular choices. Methanol has also been reported as an alternative to acetonitrile as organic solvent. However, if small percentages of methanol have shown to increase the retention time and the resolution of intact proteins, a quantity above 20% can increase the adsorption on stationary phase, even at high temperatures [128]. Besides methanol, other alcohols have been tested. Addition of n-butanol up to 5% was reported to decrease adsorption of mAbs and ADCs to the stationary phase, giving high recovery of the analytes at lower column temperature. For mAbs, besides a higher recovery at lover temperature, a reduction of the retention time is also observed. Despite all these benefits, the real mechanism with which n-butanol works is not clear yet since other related compounds (i.e., n-propanol, 2-butanol, 1,4-butandiol) are not as efficient in improving chromatographic conditions of mAbs analysis [48]. Mobile phase modifier such as trifluoroacetic acid (TFA) or formic acid (FA) are usually added to reduce peak fronting and tailing and increase resolution. Formic acid is more MS friendly, but lead to less improvements of peak resolution, compared to TFA [129]. In fact, TFA has a strong ion pairing effect that improves the retention of hydrophilic species like the peptides generated by protein digestion, although it yields to ionization suppression when MS detectors are used. Besides mobile phase composition and additives, elution gradients are also peculiar when RPLC is used for the analysis of macromolecules such as mAbs. Several studies suggest that retention of mAbs has an “on/off” mechanism. Such an hypothesis is supported by studies reporting that small changes of mobile phase composition determine a complete and sudden release of the analytes from the stationary phase. Therefore a “multi-isocratic strategy” is preferred for elution. Briefly, eluent composition is not changed gradually and linearly but stepwise, and subsequent isocratic steps at different mobile phase compositions are provided to guarantee the elution of one analyte at time[130,131]. Along with this strategy also another method called “negative gradient slope” have been reported [132]. In fact, during multi-isocratic elution, analytes with close physicochemical properties are sometimes not resolved since they co-elute from the column. For this reason, every eluent step is followed by a reverse gradient back towards the previous eluent composition at which the analytes were retained. This way a better resolution can be achieved also for species with similar partitioning.

Concerning hardware, wide pore materials are better for the RPLC analysis of big molecules such as biopharmaceuticals, as for other LC variants[48,119]. The chemistry of stationary phase has also different requirements. In terms of protein recovery, kinetic performances, selectivity, and amount of eluent modifiers needed (i.e., TFA), a high loading of phenyl moieties combined with wide pores (i.e., 400 Å) gave better results than several best-in-class stationary phases for protein analysis [47]. Surface porous silica were also reported to have low adsorption of biopharmaceuticals [133], while PEEK and titanium and new introduced materials based on ethylene-bridged siloxane chemistry were reported as alternatives for column support [134]. The use of ethylene-bridged siloxane resulted particularly good in the separation of a mixture of phosphorylated peptides, since it offers a hybrid inorganic organic surface that gives more symmetric peaks as well as higher and consistent peak areas. Autosampler hardware was also developed to overcome pre-column adsorption. Surface saturation of with BSA gave higher

analyte recovery compared to naked plastic or glass and low binding glass or plastic are currently available for protein and peptide analysis [135].

Miniaturization is also an emerging research field. Recently, stationary phase based on micromachining beds into silicon to create an array of pillars has been developed. This new kind of column have been also applied for bottom-up approach having great reproducibility and peak capacity, therefore great potential for application in biopharmaceuticals analysis [136].

Due to the variability and complexity of each analyte, several design of experiment approaches have been reported in literature to set up fast method development processes. Once identified the critical separation parameters, DoE can decrease the time necessary for a tailored method development on the single product, allowing a faster process. Moreover, analyzing a specific design space, it is also possible to gain more information than the classic one factor at the time method development. Resolution maps that are obtained from QbD experiments give more complete and informative information regarding analyte-system behavior, improving the knowledge of the system[128,137,138].

RPLC is the gold standard for the characterization of protein identity and impurities. A very popular Bottom-Up approach where the primary structure of the mAbs is determined after proteolytic digestion is peptide mapping. This approach uses trypsin or other enzymes to generate peptides that are then separated by RPLC and analyzed by tandem MS. Data analysis allows protein sequencing, which is useful to confirm the identity of mAbs but also for testing the biosimilarity of biopharmaceuticals, compared to a generator product [139]. RPLC is used also for Middle-Up experiments, and both Middle-Up and Bottom-Up approaches are used for monitoring proteoforms with post translational modifications (i.e., oxidations, truncation, and deamidation) [40]. Concerning ADCs, Middle-Up analysis is used to characterize antibody domains (e.g., light and heavy chains, Fc and Fab domains) unbound or modified with the drug payloads to achieve the characterization of the so-called isomeric Distribution of Payloads (DOP). Additionally, RPLC is widely used to analyze the small molecules of ADCs, including the amount of free drug generated by degradation or incomplete conjugation[3,11,21]. A recent paper demonstrated that an online reduction with DTT and denaturation with guanidine HCl can be performed right before the chromatographic separation. This method was used to reveal unstable antibody variants (e.g., succinimide intermediates of asparagine deamidation, aspartic acid isomerization) that are often lost when sample preparation is performed offline [140].

UV and MS are the most popular detectors for RPLC. UV detector is useful in QC process, where a known chromatographic fingerprint is expected, disregarding the level of characterization of the biopharmaceutical products (intact, Middle-Up or Bottom-Up). For this type of analyzes, any deviations from the expected chromatogram (e.g., retention time shifts, change in relative abundance of peaks), indicate the need to further analyzes by a complementary and more powerful detector, such as MS. Besides these two detectors, the application of MALS has been recently reported as useful to distinguish mAbs size variants both at Middle-Up or intact protein level based on changes in refractive index [141]. RPLC is very popular also as last step in multidimensional separations when MS detection is the goal, but the first separation is not compatible with MS. At Middle-Up level, RPLC have been used as second dimension to characterize charge variants separated by CEX [95], or glycoforms separated by HILIC [142]. Concerning the analysis of ADCs, RPLC have been used to characterize the DOP. Specifically, RPLC have been used as second dimension after HIC for cysteine-conjugated ADCs [80] and after IEX for lysine linked ADCs [94]. However, RPLC is also widely used for the analysis of small molecules. For instance, it can be used after SEC to characterize small impurities and free drug in ADCs [65]. It is also possible to use RPLC as second dimension to characterize at peptide level size variants previously separated by SEC [68]. Similarly, charge variants separated by IEX can be characterized at peptide level by multidimensional methods using RPLC as final step and MS as detector[96,97]. Interestingly, such multidimensional methods allow a complete automation including also online sample digestion and are also used for the analysis of cell culture fluids, upon Protein-A purification [111]. Bottom-Up characterization by RPLC is quite popular also at nanoflow scale and allows the characterization of glycoforms through glycopeptide mapping [143]. Notably, also RPLC-based microfluidic chips have been developed. Some chips also include sample purification or preparation steps, like glycan release via PNGase for the characterization of released IgG N-glycans by MS [144].

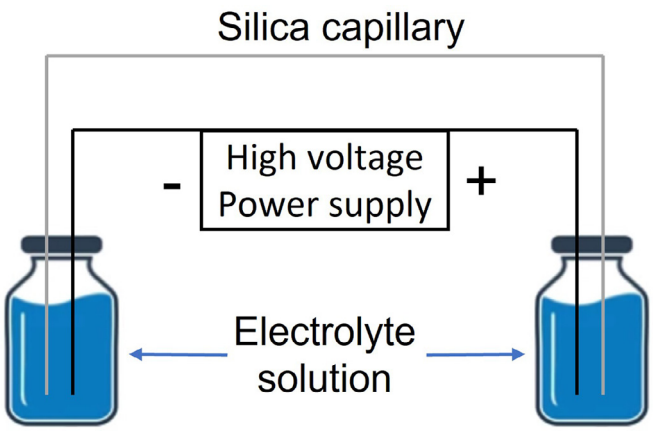

Fig. 8. Instrumental scheme of a CE apparatus.

2.1.7. *Counter-current-chromatography (CCC)*

CCC is an emerging chromatography variant applied in downstream processes of the production of biopharmaceuticals. The stationary phase is a support-free liquid, and the separation relies on a combination of liquid-liquid extraction and partition chromatography [145]. CCC is applied to continuous processes, that are characterized by a simultaneously charging and discharging of the system at uniform rates [146]. Several methodologies exists such as simulated moving beds (SMBs), periodic counter current chromatography (PCC), periodic counter current process with interconnected wash (3C-PCC/4C PCC), sequential multicolumn chromatography (SMCC), [146], and centrifugal chromatography [147]. Recently, a continuous process using a multi-column countercurrent solvent gradient purification (MCSGP) was developed for the isolation at higher yields of charge variants of antibodies for further structural characterization [33].

2.2. *Capillary electrophoresis (CE)*

Capillary Electrophoresis (CE) is a miniaturized format of electrophoresis where charged analytes are forced to migrate into a capillary under the effect of an electric field. The capillary and the two electrodes used to generate the electric field are immersed into two reservoirs containing a background electrolyte solution (BGE) as in Fig. 8.

The pH of BGE can be easily adjusted to induce either a positive or a negative net charge of proteins, therefore CE is well-suited for the analysis of biopharmaceuticals. The velocity of migration depends on the strength of the applied electric field and on an intrinsic characteristic of the analytes called mobility [148]. For proteins like mAbs and ADCs, mobility is influenced by the ratio between the analyte net charge and its hydrodynamic radius. Therefore, CE can be used to separate biomolecules owing to a different overall mobility, a different isoelectric point or a different molecular weight, since for proteins the molecular weight normally correlates with the hydrodynamic radius [149]. Although CE variants are quite diverse, the basic apparatus is roughly the same as in Fig. 8 and some common features are shared between them. Concerning sample injection, this is usually achieved by pumping the sample into the capillary (i.e., hydrodynamic injection), or by trans-

porting the sample into the capillary as effect of the electric field or by using siphoning effect (i.e., electrokinetic injection) [150].

Silica is the most popular material for CE capillaries, especially when applied for the analysis of mAbs or ADCs[22,151]. Since the analyzes are typically performed at nearly neutral pH, the inner surface of CE capillaries is negatively charged owing to the exposure of sylanols (i.e., acidic -OH moieties having typical pKa between 3 and 5). The two main issues of having a negatively charged inner capillary surface are the risk of absorbing positively charged analytes, and the generation of electroosmosis (i.e., solvent flow) along with electrophoresis (i.e., ion migration). Electroosmosis is triggered by the double layer of positive charged electrolytes adjacent to the capillary inner surface, which migrate to the negative electrode once the electric field is applied. In thin capillaries the flow of double layer ions become relevant since it drags the solution creating a net electroosmotic flow (EOF) that can be used for sample injection[150,152]. If not controlled, EOF can affect selectivity of the separation, especially when it is not homogeneous along the capillary or across different runs. EOF can be determined by measuring the mobility of a neutral compound with zero intrinsic mobility to assess its impact on separation [152]. Capillary coating is the most popular strategy to avoid protein absorption [153] and limit EOF [154]. Coating agents can be added to BGE to achieve the so-called dynamic coating [155]. This strategy relies on the establishment of a steady state equilibrium between the amount of coating agent absorbed on the silica inner surface and its concentration in BGE [155–157]. Capillary preparation is easy since loading can be achieved by a constant flow of BGE containing the coating agent through a clean capillary. Similarly, washing or replacement of the coating agent can be achieved by flushing the capillary with clean BGE or a solution containing a different coating agent. This strategy offers great flexibility for method development because many coating agents with different features have been successfully tested[155, 156, 158]. Nevertheless, there are potential limitation on the use of coating agents depending on the detector, especially because some of them are not compatible with MS [159]. Ionic coating agents allow good control of EOF, they are very soluble in BGE without altering electrical conductivity, and they are efficiently adsorbed to the capillary surface [160]. Ionic and zwitterionic agents have been successfully used for improving protein separation [161–163], while polymeric materials seem to offer better surface occupation and outperform small molecules in limiting protein capillary absorption[164,165]. Neutral compound such as polyvinylpyrrolidone can be useful in combination with surfactants to achieve an efficient coating [166], while hydrophobic polymers show a stronger absorption capacity and a complete suppression or inversion of EOF [167]. Alternatively, permanent coating of CE capillaries can be achieved both by covalent bonding or by irreversible absorption on capillary silica inner surface of either standard size or nanomaterials[168,169]. Irreversible absorption is similar to dynamic coating, although materials are not contained in BGE and they are absorbed by means of physical forces like electrostatic, hydrophobic, or hydrogen bonding interactions [170]. As for dynamic coating, the procedure is simple, flexible and does not depend on surface chemistry[157,171]. On the contrary, covalent bonding requires the chemical modification of sylanols[172,173]. Irreversible absorption gave good result for protein separation with hydrophilic compounds [170], although the best results are achieved when ionic copolymers are used [174–176]. Covalent bonding has also been successfully applied for protein analysis both by using reactive compounds [177] or by non-reactive compound that are activated by photo stimulation[178,179].

As recently confirmed, the stronger the CE electric field, the better is the peak resolution [180]. However, the higher the voltage, the higher the current passing through the BGE with a joule heating effect [150]. Heating creates temperature gradients that induce density and viscosity gradients into the capillary, as well as convection. The result are diffusion phenomena and mobility gradients that reduce the resolution [181]. The smaller the scale of separation, the higher the impact of heating on separation performance, as demonstrated for microchip-based CE separations [182]. Many strategies can be adopted to reduce such an effect, like proper modification of BGE [183], the use of carrier ampholytes [184], the use of short, thin wall capillaries to reduce current and enhance thermal dispersion [185] or the use of nanoparticle materials [186].

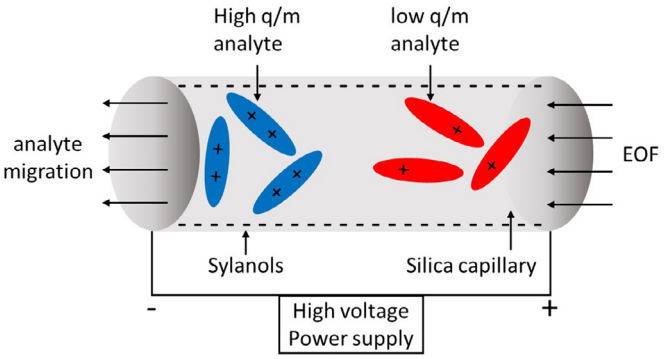

Fig. 9. Separation mechanism of a CZE capillary.

As for chromatography, CE allows the combination of orthogonal CE variants, although also hybrid CE/chromatography multidimensional separations are possible. Moreover, like for chromatography, some CE approaches can be developed using conditions that either preserve or induce a denaturation of the structure of biological molecules. These features make CE well suited for the analysis of some important CQAs of biopharmaceuticals[2,3,11,22,151,187].

2.2.1. Capillary zone electrophoresis (CZE)

CZE is the simplest and the most straightforward CE variant and since its first application in 1981 [188] remained the most popular form of CE. Analytes are separated owing to their different velocity of migration that correlates to intrinsic mobility, which for proteins depends on charge to mass ratio (see Fig. 9) [189]. However, a different velocity of migration can be observed also as function of the electric field applied and EOF generated during the analyzes. Such parameters must be therefore controlled during separations.

As extensively reported, CZE can be used for the identification of charge variants of mAbs and ADCs [2]. In fact, many post translational modifications (e.g., deamidations, oxidations, glycans heterogeneity) have on one hand negligible impact on molecular weight, while on the other hand can induce a significant shift of the isoelectric point that influence the charge of the analytes during CE separation [2]. Since the resolution of charge variants is obtained owing to the pI differences between species, the pH of BGE is one of the parameters requiring a fine tuning during method development, since it determine the net charge of the analytes during the separation[190,191]. Moreover, other BGE flows triggered by EOF or convection phenomena must be minimized since mobility should depend mainly on analyte net charge. EOF and joule heating can be suppressed by adding BGE modifiers to reach the so-called dynamic coating[191,192]. Alternatively, good results can be achieved also by static capillary coating [190] or some other innovative strategies, one of which allowed to achieve a fibrin coating by an in-situ polymerization triggered by thrombin catalysis, as for blood coagulation [34]. Coating is also useful to reduce absorption on capillary wall and allow to use stronger electric fields and longer capillaries with further resolution improvements [180]. Such improvements have proven to allow separation of charge variants also for mixtures of different mAbs [193].

CZE methods for the determination of antibody charge variants can be validated both for antibodies and ADCs [194], and were proven to be very robust since methods can be developed to comply GMP specifications [195]. However, although the method setup is fast and relatively simple, CZE does not compare with CIEF nor with IEX for the separation of charge variants. Specifically, by comparing methods based

on fluorescence detectors, CZE was outperformed in term of precision, separation efficiency or sensitivity [27]. CZE can be developed in microfluidic and on-chip systems. Such methods are sometimes reported as microfluidics zone electrophoresis (MZE) and they demonstrated to be suitable for high throughput screening of charge heterogeneity and impurities of antibodies, with shorter analysis time [196–198]. Moreover, microfluidics CE allows to perform separation of antibody charge variants with results comparable to CIEF [199]. Besides intact antibody analysis, some recent applications of CZE are focused on Bottom-Up and Middle-Up methods. CZE was successfully applied for the separation of the main proteoforms of antibody substructures (i.e., light and heavy chains) obtained after chemical degradation with reducing agents [200]. CZE was used also for the separation of Fc/2 and F(ab')2 fragments obtained from IdeS digestion, allowing the characterization of lysine mutations and N-glycosylation variants [201–203]. The information obtained from the analysis of intact and partially degraded monoclonal antibodies have been found to be complementary to characterize the so-called proteoforms [204]. The interest in using CZE for Bottom-Up studies comes from the goal of finding an alternative to RPLC for standard peptide fingerprinting methods. Several papers described the successful application of CE for the analysis of the primary structure and post translational modifications of antibodies and ADCs at peptide level, after enzymatic degradation [205–208]. Microfluidics CZE was also developed for peptide mapping [199]. A recent study highlighted that CZE outperforms standard chromatographic methods for antibody sequencing [209]. Interestingly, the study also reported that CE and liquid chromatography give complementary information allowing complete sequence coverage of novel antibodies. Importantly, antibody disulfide reduction and trypsin degradation were recently implemented directly into the capillary, by mixing the reagents through transverse diffusion of laminar flow profile (TDLFP)[210,211]. Peptide level analysis opened new perspectives for the use of CZE for the characterization of other CQAs such as the identification of protein impurities, namely host cell proteins (HCPs). Compared to other separation techniques commonly used for the identification of HCPs, CZE works well also without antibody depletion[212,213]. Moreover, CZE was also used after in solution[35,214,215] or solid phase supported [216] offline PNGase F digestion to map human IGg glycoforms. Interestingly, CZE was recently used also for monitoring of pool energy expenditure of host cells during antibody production [217].

UV is the most popular detector for the analysis of charge variants by using CZE[2,22,151,187]. Compared to CIEF, CZE allows the use of BGE with less UV noise, resulting in a considerably higher sensitivity at lower wavelengths [218]. Besides UV, laser induced fluorescence (LIF) and mass spectrometry (MS) are the other detectors described in literature[2,22,151,187]. The most popular derivatizing agents both for antibody and glycan analyzes is 8-Aminopyrene-1,3,6-trisulfonic-acid (APTS)[35,216], which is used for fluorescence detection but it is also compatible with MS, if appropriately used [219]. Derivatization with stable isotopes of 2-aminobenzoic acid (2-AA) have been also used to obtain orthogonal information from the analysis on the same product using both CE-MS and LC-MS [220]. CZE coupled with MS detector is well suited for keeping the antibodies in native form [221]. This is very important for minimizing the artifacts caused by product de-structuration during the analyzes. CZE-MS allows Middle-Up approaches that are complementary to intact molecule analyzes [204]. MS coupling is usually performed with ESI[22,151,187]. The main barrier for CZE-MS hyphenation is the BGE composition. One popular setup consists of MS compatible salts and a conductive sheath-liquid, connected to the outlet electrode of the CE to maintain the electrical field and a stable spray [222]. However, CZE-MS hyphenation can be done also by using sheathless configurations [223]. Recently, a dynamic pH barrage junction was developed to enhance the sensitivity of amino acids, peptides, and digested monoclonal antibodies in CZE–MS [224]. 3D printed interface for MS have been also developed to improve the analysis performances by maximizing Joule-heating dissipation [225]. Microfluidic devices with integrated ESI have been also developed for antibody analysis [199]. Such miniaturized systems required the development of peculiar coating strategies to enhance the separation performances without affecting MS detection [226]. For microfluidics and chip systems coating is essential for minimizing the joule heating effect that has a bigger impact on such miniaturized separations [182]. Since CZE can be used as an alternative to chromatography for peptide mapping experiments [209], a recent paper aimed at developing innovative methods for mapping host cell impurities in antibodies demonstrates that LC-MS/MS and CE-MS/MS techniques often produce orthogonal results. Interestingly, the combination of the two separation techniques (LC–CE-MS/MS) combines the benefits of them and allow the identification of peptides in a wider range of size, pI, and hydrophobicity [227]. Hybrid separation relying on both CZE and LC, have been also achieved with microfluidic devices that allow an easy hyphenation with MS and further demonstrate the complementarity of such separation techniques [228]. CZE-MS is widely used also for the analysis of antibody glycans[214,215] and recently the benefits of using cutting edge MS instrumentation with drift tube ion mobility system was reported. The study highlighted the advantages of ion mobility mass spectrometry for the analysis of native and APTS-labeled N-glycans [229]. Since CZE allows MS detection, it is widely used as bridge between MS and other CE variants not allowing a direct coupling with such detector. In this context CZE have been used after SDS based capillary gel electrophoresis [230] but also after capillary isoelectric focusing[231,232], to allow MS detection. However, two dimensional CZE separation of antibodies have been also described as a configuration allowing to maximize the separation of charge variants [233]. In fact, the optimization of CZE separation often requires the use of BGE additives not compatible with MS. To achieve the better separation with MS detection a first CZE separation can be performed using a method not compatible with MS detection, while a second CZE in MS friendly conditions can be used as a bridge between MS and the first dimension. CZE coupling with other techniques is also used to improve loading capacity or sample pre-purification. Specifically, some papers about the use of CZE before capillary isoelectric focusing [232], isotachophoresis (ITP) [234] or inline solid phase extraction [235] have been reported.

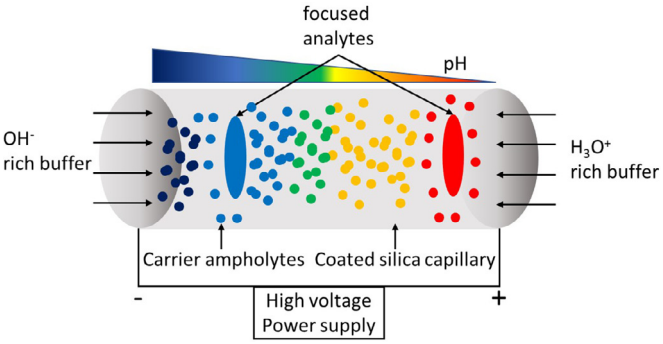

Fig. 10. Separation mechanism of a cIEF capillary.

2.2.2. *Capillary isoelectric focusing (CIEF)*

CIEF is a separation technique based on the resolution of analytes having different isoelectric points and was derived as an adaptation of the early forms of CZE [236]. CIEF relies on the generation of a pH gradient into the capillary, so the analytes migrate until they have a net charge. This way the molecules can be focused to specific zones of the capillary where the pH of the surrounding solution equals their pI [236]. The capillary is placed between two electrodes immersed into two vials. An acidic solution is placed at the anode (anolite) and a basic solution (catholite) at the cathode. The electric field starts the migration of hydronium ions from the anolyte to the cathode and hydroxide ions from the catholyte to the anode (see Fig. 10).

To achieve a buffered pH gradient into the capillary, carrier ampholytes are loaded into the capillary with the sample. Carrier ampholytes are molecules containing both acidic and basic moieties with a strong buffering capacity [237]. Each ampholyte migrates and focus to a different capillary zone and then it buffers the surrounding solution to a specific pH. Carrier ampholytes have been recently implemented also on CZE, generating a new CE variant called Carrier Ampholytes-Based Capillary Electrophoresis (CABCE), which showed better performance of CZE in separation of protein mixtures. However, no application for biopharmaceuticals have been reported so far [184]. In CIEF, a decrease of current is observed under a constant electric field, since the ions stop migrating once focused. The signal of the end of focusing is therefore a low plateau of current. Alternatively, focusing can be done at constant current flow, increasing the voltage to compensate the reduction of migrating ions, until a high voltage plateau is reached [150].

To obtain the best results, CIEF requires silica coating, since the presence of a EOF continuously dragging the analytes makes impossible the focusing of the analytes. For this reason, CIEF capillaries are typically coated by using linear polyacrylamide polymers (LPA) [2], hydroxypropyl methylcellulose (HPMC) [197], or PVA [238]. Over the years several strategies have been reported to allow a better neutralization or burying of charged sylanols [239]. As already described for CZE, coating is also useful to minimize joule heating and protein absorption. However, protein absorption can only be reduced and not eliminated. Recent studies reported therefore that an increase of precision can be reached after implementation of rinsing steps for the removal of proteins absorbed from previous analyzes [240–242]. Besides coating agents, many other additives are requested in CIEF. Concerning anolytes and catholytes, H_3PO_4 and NaOH are the most popular compounds, while proprietary composition mixtures of various poly amino carboxylic acids are typically used as carrier ampholytes [2]. Recently, some innovative technologies have been developed to create capillaries with immobilized pH gradients for the measurement of pI. Immobilization was performed by chemical or radical reactions with epoxy or acrylamide groups, after the focusing of carrier ampholytes into the capillary. Monolith materials for the support of carrier ampholytes can be created in situ by polymerization reactions[243,244]. Alternatively, silica capillaries can be coated by the covalent reaction of sylanols with acrylamide derivatives [245]. A third strategy is the creation of CIEF columns through the packing of silica capillaries with modified silica particles that expose epoxy groups used to covalently bind carrier ampholytes after a focusing step [246]. Other additives for CIEF are sacrificing agents, which are amphoteric compounds able to stabilize the pH gradient and avoid anodic or cathodic drifts of analytes and carrier ampholytes caused by ITP, which happens simultaneously to IEF [247]. The use of solubility enhancers is particularly important in CIEF since proteins like biopharmaceuticals are less soluble at their pI values. Urea, glycerol, acetonitrile and detergents are the most popular solubility enhancers [2], although recently also DMSO have been reported [248]. A potential problem of using such reagents is protein denaturation. On one hand this does not allow to study analyte properties in native conditions, on the other hand denaturation can undergo aggregation. Since protein integrity maintenance is very important, some authors published a study about non-detergent additives that stabilize charge and solubility without causing aggregation [249].

Although the analysis of charge variants of biopharmaceuticals can also be performed with other techniques such as IEX or CZE, CIEF is still considered the gold standard [92]. Concerning ADCs, a recent paper compared the influence of conjugated linker-drug on the separation of charge variants, concluding that such structural modifications affects separation by IEX but not by CIEF [93]. It was also reported that CIEF outperforms both IEX and CZE in term of peak resolution and precision of pI determination [27], which is achieved by comparing the focusing of samples and reference standards [2]. The wide scale applicability of CIEF was demonstrated by a study reporting the characterization of pI and the charge variants of 23 therapeutic mAbs [250]. Robustness of CIEF was demonstrated by the validation of an interlaboratory method for charge heterogeneity analysis of mAbs[251,252] and other study on the qualification of NISTmAb as reference standard [218]. Biopharmaceutical biosimilarity of different products can be assessed by CIEF through the characterization of charge heterogeneity [253], alone or in combination with product stability assessment [254]. Middle-Up analyzes for the characterization of domain-specific charge variants have been reported as well [255]. Moreover, since CIEF gives complementary information compared to other CE variants, a comprehensive antibody characterization can be achieved by parallel analysis with different CE variants, including CIEF for the characterization of charge variants [256]. CIEF have been also used for monitoring the stability of antibodies in infusion solutions. Both IEX and CIEF were able to detect dextrose-glycated antibodies, but unlike CIEF the overall glycation was underestimated using IEX, since labile Schiff base were disrupted during the analysis [257]. The non-capillary version of isoelectric focusing (IEF) was also used in combination with free-flow electrophoresis (FFE) for the collection of isolated charge variants of antibodies in quantities sufficient for further analysis [36]. CIEF is also very popular for the analysis of bioconjugates such as ADCs. For instance, lysine-linked ADC typically have pI shifts due to lysine modifications, so CIEF is well suited to map their heterogeneity[258,259]. The stability of the conjugation is another concern for ADC, therefore some authors reported a specific investigation on the chemical stability of some linkers [260], while other authors used CIEF for the setup of stress tests for the assessment of product storage stability [261]. Microfluidic and on-chip versions of CIEF have been also described for short time antibody analysis with smaller consumption of sample and solvents[262,263].

Analyte detection can be performed by using two different approaches. Historically, the first approach was to force sample migration to the detector (i.e., mobilization) upon focusing was complete. Analyte mobilization can be achieved either hydrodynamically or chemically. Pressure or vacuum are the common driving forces used for hydrodynamic mobilization, while chemical mobilization is achieved by replacing the catholyte or the anolyte to disrupt the pH gradient and force a net charging of the analytes, which induces their migration towards the detector [2]. UV is the most common detector for mobilization-based methods. However, detection cannot be performed at low wavelengths due to the interference of carrier ampholytes[2,22,151,187]. Recent examples of CIEF with mobilization and detection have been reported in native condition both for the analysis intact mAbs or for Middle-Up experiments [264]. However, the most popular detection strategy in CIEF is the whole capillary imaging[2,22,151,187]. In imaged CIEF, UV-detection is performed by using a charge coupled device camera (CCD) that scan all the capillary after focusing, without analyte mobilization. This procedure is fast and avoid peak broadening associated with mobilization [22]. Imaged CIEF can be used also for bidimensional separation. It was reported as second dimension for the characterization of antibody deamidation after a first separation using HIC [265] or IEX [266]. The data obtained by imaged CIEF are complementary to data obtained with other techniques able to detect single amino acid modifications (e.g., chromatography-based peptide mapping), so CIEF can be used for a multistage analysis of antibody charge variants [266]. Fluorescence is not so popular as for other CE variants. Nevertheless, it has been reported that CIEF is more sensitive than both IEX and CZE when used to analyze charge variants detected by means of antibody native fluorescence [27]. Direct coupling of CIEF with MS is also difficult because of the poor compatibility between CIEF additives and ionization techniques such as ESI [267–269]. However, some papers describing direct CIEF-MS coupling have recently been published. Some authors replaced popular catholytes and anolytes with MS friendly compounds, using glycerol as extra additive. A neutral capillary was also placed between regular CIEF and MS to mitigate ionization suppression [270]. Other common strategies that have been reported are the use of sheath acidic liquids, immobilization of carrier ampholytes on monolithic materials or the development of methods using diluted additives [271].

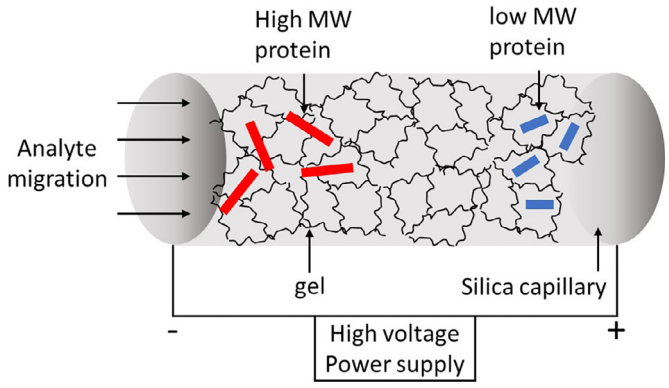

Fig. 11. Separation mechanism of a CGE capillary.

Miniaturization on chip makes also possible a direct MS hyphenation of imaged CIEF [263]. An interesting paper has reported the setup of an imaged CIEF configuration allowing the hyphenation with MS through a four port nanoliter valve allowing to setup a 2D system to cut the focused analyte and transfer them to a second dimension for a CZE-MS analysis [272]. Another paper reported some advantages of setting up a CIEF assisted CZE-MS platform, like an increase of the loading capacity and better resolution of antibody proteoforms, compared to standalone CZE-MS [232].

2.2.3. Capillary gel electrophoresis (CGE)

CGE is a variant of CE where silica capillary is filled with a gel matrix. When migration occur through the gel, mobility is therefore influenced mainly by the higher frictional forces experienced by the molecules (See Fig. 11).

Polyacrylamide gels are the most popular [247], although the traditional slab gels used for planar gel electrophoresis (i.e., SDS-PAGE) are replaced by soluble polymers used as replaceable molecular sieve [151]. A recent study demonstrated that for antibody analysis the smaller the mass differences between species, the higher the influence of gel density (i.e., monomer to crosslinker ratio) on separation performance [273]. Separation of proteins can be achieved with or without disulfide reducing agents, while SDS is the most used detergent added to cover proteins and shift the net charge of all molecules to a negative value. This neutralizes the effect of charge differences that may occur between the analytes, and migration is therefore influenced only by the size of the molecules [187]. The use of detergents other than SDS have been recently reported to enhance the performance of the separation both in non-reducing [274] and reducing conditions [275]. As for other CE variants, EOF must be suppressed as much as possible to avoid migration drifts. Linear polyacrylamide (LPA) [276], hydroxypropyl cellulose (HPC) [277], polyethylene glycol dimethacrylate (PEGDMA) [278], and Polyvinyl alcohol (PVA)[279,280] are the most common coating agents added to BGE. Alternatively, some branded BGE containing mixture of coating agents are available from vendors [151]. As already described, coating has also a positive impact on minimizing peak drifts due to capillary heating that generates convection [281].

CGE is used for the characterization of a wide array of CQAs of biopharmaceuticals, including the assessment of biosimilarity, antibody stability and aggregation, purity, size heterogeneity and N-glycan isoforms, but also drug-to-antibody ratio DAR of ADCs[22,151]. In general, CGE outperforms the corresponding planar technique in term of both accuracy and resolution of antibody size variants [282], although some recent studies demonstrate that fine tuning of planar technique can revert such a trend [283].

Since CGE separates molecules according to their size, it is well suited to detect impurities or degradation products generated by covalent aggregation [284] or fragmentation [285] (i.e., size variants). The results obtained by CGE are consistent with those obtained by SEC [286]. Several antibody standards like NISTmAb have been tested to demonstrate the possibility of standardizing CGE protocols for size heterogeneity testing [287]. In some studies, CGE resulted to be robust enough to pass interlaboratory validation [288], although other studies reported some concerns about the generation of artifacts during the analyzes as result of baseline drifts [289], antibody fragmentation [290], poor SDS-induced denaturation [291], iodoacetamide non-specific alkylation [292] or noncovalent dimerization [293]. Concerning stability studies, the comparison between CGE results obtained in reducing and non-reducing conditions has been proved to be useful for antibody analysis [294]. Several other studies following such procedure are available on literature[22,151]. Recently, CGE was applied to investigate antibody stability in host cells during production, with a particular focus on fragmentation side reactions [285]. CGE is used also for the analysis of bioconjugates such as ADCs. One attribute that can be determined by CGE is the purity, compared to unconjugated antibody. This is important to demonstrate that conjugation does not generate antibody fragments[11,295]. For cysteine modified ADCs, CGE have been used to determine the payload distribution and positional isomers (DOP) [296]. CGE has been used for biosimilarity studies between innovator and commercial antibodies [297] or ADCs [298]. Middle-Up analyzes can be performed by using proper gel densities. CGE can be used to monitor the heterogeneity of heavy and light chains after partial degradation with reducing agents [273], or for the analysis of glycoforms after degradation with de-glycosylating enzymes. As for other liquid phase separation, the analysis of glycans requires derivatization with fluorescent probes [299]. The analysis of glycans can be used for high throughput screening [300] and PNGase F can be immobilized into microcolumns for automated N-glycan release [301].

CGE is complementary with other CE variants such as CIEF, but also with some LC variants. For instance, it was reported that CGE is useful to detect impurities that can be further identified by LC-MS/MS [302]. Since CGE is useful to characterize size variants and glycoforms, while CIEF is used for the characterization of charge variants, a multilevel antibody characterization can be achieved by parallel analysis relying only on different CE approaches [256]. CGE is also scalable to in-chip miniaturized formats tht reduce analysis time and sample consumption [303–305]. However, for SDS based separation, it was reported that to optimize the separation a fine tuning of the amounts of surfactant is needed when the analysis is scaled down [304].

As already for other CE variants, UV is the most popular detector. For protein detection is usually performed at low wavelengths since CGE additives do not interfere[2,22,151,187]. Similarly, LIF is a very popular detector. Released glycans can be analyzed upon derivatization with APTS[300,306,307], 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS) [306] or 2-AA [308]. Recently, 3-(2-furoyl)-quinoline-2-carboxaldehyde (FQ) was used for antibody derivatization and size heterogeneity assessment at intact mAbs level [309]. Finally, Teal^{TM} is a recently reported compound that allows MS hyphenation and provides high performance and reproducibility even at low concentration [310]. MS detection remains incompatible with SDS based CGE, since SDS interferes with ionization. Recently, the in-capillary co-injection of positively charged surfactants and methanol as organic solvent have been reported as an appealing strategy to neutralize SDS and allow a direct coupling of SDS-based CGE with MS [311]. Alternatively, CZE can be used as second dimension to allow online hyphenation of MS with SDS based CGE [230].

2.2.4. Affinity capillary electrophoresis (ACE)

Affinity capillary electrophoresis (ACE) is a variant of capillary electrophoresis where analytes reversibly interact with one or more components of the system during migration. Since the interaction can be performed in several different ways, ACE refers to a family of analytical approaches, rather than a defined method [312]. A variant defined Flow-Through Partial-Filling Affinity Capillary Electrophoresis (FTP-FACE) has been recently applied for the analysis of a mixture of mAbs. To characterize the charge variants of one of the two co-formulated an-

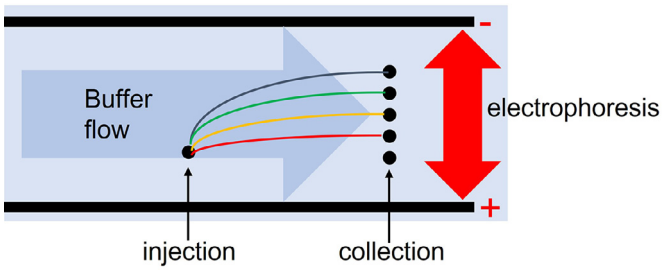

Fig. 12. Instrumental scheme of a FFE apparatus.

tibodies, a specific ligand was added to induce the migration shift of the other antibody that was interfering with the analysis. The separation principle driving the separation of the charge variants is the same of CZE [37].

2.2.5. Free flow electrophoresis (FFE)

In FFE molecules are separated in a buffered solution owing to both a hydrodynamic flow and an electric field [313]. The buffer flows through a chamber build between two plates and a laminar flow is ensured by a peristaltic pump. Two electrodes are applied to ensure an electric field orthogonal to the buffer flow (see Fig. 12). The sample is injected on one corner of the plate and while it is transported by the buffer flow, an electrophoretic migration occurs orthogonally. FFE is available in miniaturize format and any of the CE variants described above can be used in the direction of the electric field, to ensure the best separation of the molecules in a two-dimension space. FFE is very versatile since both buffer flow and electric field can be turned on and off at will to develop many different applications [313]. However, FFE was only recently applied for antibody analysis by using IEF mode. In detail, FFE has been used for the fractionation and collection of charge variants that can be then characterized by MS[36, 38]. Interestingly, the separation efficiency of FFE resulted to be comparable to standard CIEF, with the advantage of achieving sample fractionation of relatively high amounts of proteoforms. This is an important result since recovery limits the application of some analytical techniques for the characterization of impurities of biopharmaceuticals.

2.3. Other electrokinetic techniques

2.3.1. Capillary electrochromatography (CEC)

CEC is a variant of chromatography having an electrokinetically driven flow of the eluent [314]. Unlike LC, electroosmosis is used to induce the permeation of the eluent through the stationary phase. An electroosmotic flow can be induced by applying an electric field by means of two electrodes, as for CE. For this reason, CEC is considered a hybrid between LC and CE. The application of CEC for the analysis of biopharmaceuticals has increased in the past decade [315]. Concerning antibody analysis, two papers reported CEC application for the characterization of CQAs. Specifically, the separation of charge variants was achieved in open tubular capillaries coated with bovine serum albumin (BSA) [39]. The protein was immobilized in two steps consisting of silica capillary coating with poly-dially-dimethylammonium chloride (PDDA), followed by BSA binding on coated capillary. At pH 6 a good resolution of the charge variants of mAbs was achieved, while increasing or decreasing the pH affected separation by altering either the mobility of charge variants or the interaction with BSA-coated stationary phase. Similarly, thrombin was used as a catalyst to induce capillary coating by in situ polymerization of fibrin [34]. The so-prepared capillaries were used to separate the same samples analyzed by BSA-coated capillaries [39], and a similar pH dependence of the performances were reported.

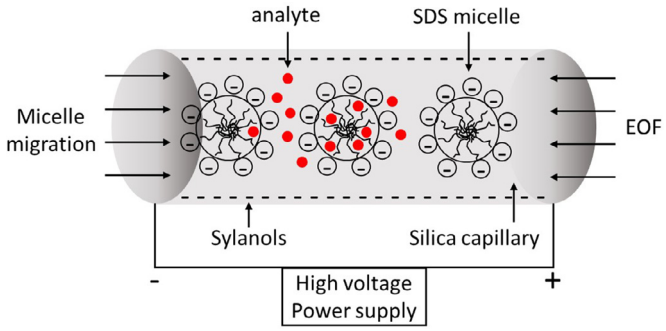

Fig. 13. Separation mechanism of a MECK column.

2.3.2. Micellar electrokinetic capillary chromatography (MEKC)

MECK is an analytical technique with a setup resembling CE. Micelles are generated by adding a surfactant (usually SDS) into BGE up to its critical micellar concentration. A proper BGE composition is used to generate a strong EOF towards the negative electrode. Since SDS is negatively charged, micelles migrate towards the positive electrode but are curbed since EOF is flowing the opposite way (see Fig. 13). Analytes are separated according to their partitioning between the hydrophilic and hydrophobic phases (i.e., BGE and SDS micelles, respectively). The mechanism of separation is borderline between CE and RPLC and some authors described the separation mechanism of proteins as a particular case of ACE (surfACE), if SDS is used to create micelles [277]. Few applications of MECK have been reported for the analysis of both glycoforms and charge variants. MECK showed some interesting orthogonal results compared to CZE when applied for the characterization of human IgG glycosylation, with the benefit of allowing larger peak capacity [35]. Concerning the analysis of charge variants, a comparative study among different separation methods reported that MECK performed as good as CZE, but with less resolution than IEX and CIEF [27].

3. Conclusion

Liquid chromatography and capillary electrophoresis are two families of separation techniques that have been extensively used for the characterization of biopharmaceuticals. Over the years, many variants have been developed using many different physicochemical principles to drive the separation of the analytes. Even some hybrid techniques such as capillary electrochromatography or micellar electrokinetic capillary chromatography have been developed to include the most attractive features of both liquid chromatography and capillary electrophoresis in a single analysis. Each separation technique looks suited for monitoring specific CQAs of mAbs and ADCs. Therefore, the characterization of bioconjugates requires the use of multiple approaches based on orthogonal separation principles. This can be achieved by separate analyzes aimed at characterizing different CQAs, or within a single run through the setup of multidimensional separations. Beside multidimensionality, the state of the art of the actual research is focused also on automation, improvement of material performances and miniaturization of the instruments to reduce costs and analysis time. However, the efforts done in the technology development need to be paralleled by regulatory aspects that are still missing. One critical issue is the availability of certified quality control standards, but also of accurate and comprehensive validation guidelines for the different analytical methods that have been developed. These two aspects are crucial for the and harmonization of the results obtained across laboratories, across different instruments or by using orthogonal analysis methods.

CRediT authorship contribution statement

Ettore Gilardoni: Writing – original draft, Writing – review & editing, Conceptualization. **Luca Regazzoni:** Writing – original draft, Writing – review & editing, Conceptualization, Supervision.

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