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New analytical challenges in characterization of antibody-drug conjugates

Erika Maria Ricci [a,b], Enrico Dainese [c], Vincenzo De Laurenzi [d,e], Giulio Lovato [d,e], Gianluca Sala [d,e], Marcello Locatelli [a,*], Miryam Perrucci [c,f]

[a] Department of Science, University of Chieti-Pescara "G. d'Annunzio", 66100 Chieti, Italy
[b] Department of Medical, Oral and Biotechnological Sciences, University G. d'Annunzio, Chieti- Pescara and Center for Advanced Studies and Technology – CAST, 66100 Chieti, Italy
[c] Department of Biosciences and Agro-Food and Environmental Technologies, University of Teramo, 64100 Teramo, Italy
[d] Department of Innovative Technologies in Medicine and Dentistry, Center for Advanced Studies and Technology (CAST), University of Chieti-Pescara "G. d'Annunzio", Chieti 66100, Italy
[e] Center for Advanced Studies and Technology (CAST), University of Chieti-Pescara "G. d'Annunzio", Chieti 66100, Italy
[f] Department of Innovative Technologies in Medicine & Dentistry, University "G. d'Annunzio" of Chieti-Pescara, 66100 Chieti, Italy

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ABSTRACT

Since 1913, when Paul Ehrlich introduced the concept of "selective toxicity", new strategies to obtain quality control, quantification of the drug were mandatory. For this reason, the introduction of antibody drug conjugates (ADC) has shown many critical points, being these systems made of small- and high-molecular weight compounds. Being ADCs a novelty for showing therapeutic action, they were subjected to a grow up in studies. There are in literature several approaches to follow ADCs for *in vivo* and *in vitro* studies because type of conjugation, release of the drug, and body-distribution are characteristic for each of them, resulting in many critical steps.

Liquid chromatography (LC) and capillary electrophoresis (CE) are the most common analytical technique used in order to obtain the aim. For the characterization of ADCs, there are several chromatographic techniques. HPLC finds application in both small and large molecules, related to the availability of various modes of separation (reversed-phase, size exclusion, ion exchange, mixed-mode etc.). Capillary electrophoresis (CE) finds its main application in large molecule therapeutics, where its electrophoretic separation mechanism offers a distinct, and often superior, separation of macromolecules compared to classic chromatographic techniques. Several modes of CE, including capillary electrophoresis sodium dodecyl sulphate (CE-SDS), capillary zone electrophoresis (CZE), and capillary isoelectric focusing (CIEF) or imaged capillary isoelectric focusing (iCIEF) are commonly utilized in characterization of the critical quality attributes (CQAs) of monoclonal antibody drugs such as charge variants, size variants, and positional isomers/purity etc.

In this review, analytical methods for physicochemical characterization of ADCs will be reported and analysed in order to highlight as the chromatographic procedures allow obtaining a complete ADCs evaluation and characterization.

1. Introduction

According to the contemporary definitions, cancer is defined as a pathological condition marked by the unregulated proliferation of transformed cells that are subject to evolutionary pressures through natural selection. Furthermore, it is now well-established that genetic mutations serve as pivotal initiating factors in oncogenesis [1]. Recent data indicate that more than 19.3 million new cancer cases were diagnosed, leading to around 10 million deaths in 2020 [2]. Chemotherapy remains one of the most prevalent treatment modalities for cancer, employing cytotoxic agents to eliminate malignant cells or inhibit their proliferation. However, due to its non-selective nature, chemotherapy often adversely affects other rapidly dividing healthy cells, such as those of the hair follicles, gastrointestinal tract, and bone marrow [3]. In order to limit these side effects and gain the maximum efficiency of action, it is essential to develop new strategies while avoiding healthy cells. This concept can be summarised in the expression of "magic bullet" introduced by Paul Ehrlich, the founder of chemotherapy [4].

Inspired by the "magic bullet" paradigm, molecular targeted therapy also referred to as "Personalized Medicine" has emerged as a pivotal

* Corresponding author.
E-mail address: marcello.locatelli@unich.it (M. Locatelli).

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approach in oncology. This strategy involves the use of pharmacological agents or other bioactive substances specifically designed to interact with defined molecular targets implicated in tumour development and progression. These targets are typically molecules that are differentially expressed or functionally dysregulated in cancer cells compared to normal tissues. Molecularly targeted therapeutic agents exhibit a broad spectrum of mechanisms of action and pharmacodynamic profiles [5–7]. Molecular targeted therapies achieve anticancer effects through various mechanisms, such as inhibition of cell proliferation, metastasis, and angiogenesis, induction of apoptosis, and reversal of multidrug resistance [8,9].

One of most innovative approach for oncology therapy is the use of Antibody Drug Conjugates (ADCs), which pivotal aim is matching the selectivity of monoclonal antibodies with cytotoxic drugs, small molecules useless alone for their high toxicity [10]. ADCs consist of three primary components: a monoclonal antibody, a cytotoxic payload, and a linker that connects the two (Fig. 1). Different conjugation chemistries are employed; the most common methods involve linking through lysine residues (ε-amine group, -NH_2 in its deprotonated form) or cysteine residues (sulfhydryl group, -SH). The antibody is chosen based on its ability to specifically recognize a molecular target, prioritizing those with the highest affinity and selectivity. Ideally, the antibody should bind exclusively to a target that is overexpressed on tumour cells to minimize off-target delivery of the therapeutic payload to healthy tissues. The target antigen on the cancer cell should be abundantly expressed (greater than 10^5 copies per cell) and must be bound by the antibody with a strong affinity ($K_d \leq 10$ nM) to ensure efficient internalization into the target cell [11].

In the first generation of ADCs, murine antibodies were often recognized as foreign by the human immune system, provoking a robust immune response and the production of anti-mouse antibodies, which compromised their therapeutic efficacy. This limitation was partially overcome in second-generation ADCs through genetic engineering that produced chimeric antibodies combining murine and human components. Although these chimeric ADCs showed promising results in cancer therapy, challenges such as diminished effectiveness and the induction of human anti-chimeric antibodies sometimes persisted. To further improve immunocompatibility, significant efforts have been made to develop humanized monoclonal antibodies, which retain only the murine complementarity-determining regions (CDRs) within a human variable region framework, or fully human antibodies [12]. One of the fundamental points of conjugation chemistry is the precise control of the Drug-to-Antibody Ratio (DAR). Chosen antibodies and their corresponding ADCs can target antigens that either trigger or do not trigger internalization via receptor-mediated endocytosis (RME). Based on this characteristic, ADCs are categorized as either internalizing or non-internalizing [10].

In internalizing ADCs, the antibody performs a fundamental role as it favours the internalization of the target antigen receptor. Internalizing ADCs enter target cells via receptor-mediated endocytosis, a process driven by the antibody's role in receptor internalization. After uptake,

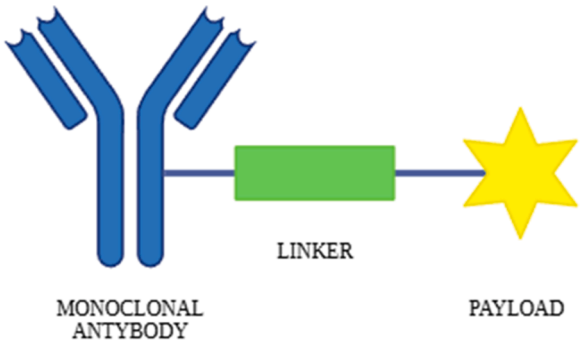

Fig. 1. ADC general composition.

ADCs travel through endocytic pathways, primarily clathrin-mediated endocytosis, where they are processed in acidic endo-lysosomal compartments. This environment triggers ADC degradation and drug release, allowing the payload to reach the cytosol and kill cancer cells through specific mechanisms [13]. Non-internalizing ADCs also rely on extracellular drug release within the tumour environment, where the cytotoxic payload diffuses into and kills target and nearby tumour cells, amplifying the therapeutic effect through a bystander mechanism. Bystander effect is that property of some ADCs, which exhibit their cytotoxic activity against cancer cells near the ligand target. It is based on linker and payload, and bystander effect was studied to improve activity for heterogeneous tumors [14]. Many studies have reported that to improve bystander effects some characteristics as cleavable linker and hydrophobic payload are almost necessary [15–17].

ADCs rely on either cleavable or non-cleavable linkers to connect the drug to the antibody. Cleavable linkers enable payload release in either the tumour environment or inside cells, making them suitable for both internalizing and non-internalizing ADCs [18]. Non-cleavable linkers require internalization and intracellular degradation to release the drug, limiting their bystander effect potential. Linker choice is essential for drug stability in circulation and effective, targeted payload delivery. The most used non-cleavable linkers are alkylic and polymeric while among cleavable linker there are: disulphide linkers, cathepsin B-sensitive linker, hydrazone linker and Glycosidase-sensitive Linkers. Some *in vivo* bio- reaction are summarized in Fig. 2 based on chemical structure of linkers. About chemical cleavable linker, there are two main class, hydrazone- and disulphide bond. In the first one, the C- terminus of the hydrazone linker is conjugated to cysteine of mAb though an acetylbutyrryl group and hydrazine is conjugated to the payload. Instead, one (or more) sulphur atom is obtained by disulfide bridge of the cysteine (mAb) and the other from the thiol group of the payload. About enzumatic cleavable linker, mainly conjugation are based on Val- Cit and Val- Ala peptide liker, where NH_2 is conjugated with cysteine residues and COOH to cytotoxic agent [19].

Currently, most ADCs are designed using two main classes of highly toxic compounds that target either microtubules or DNA. The first class includes auristatins and maytansines, which act as tubulin inhibitors and have been extensively utilized in ADC development. The second class comprises DNA-damaging agents such as calicheamicins and pyrrolobenzodiazepines (PBDs), which induce apoptosis in all cells, including cancer stem cells (CSCs). Ideal ADC payloads must possess several key characteristics: *(a)* good aqueous solubility to facilitate conjugation and maintain ADC stability under physiological conditions; *(b)* extremely high cytotoxic potency, with IC50 values typically between 0.01 and 0.1 nM, surpassing that of standard chemotherapeutics; *(c)* the ability to induce cancer cell death primarily through apoptosis; and *(d)* appropriate functional groups to enable effective attachment to the antibody [20]. In Table 1 are reported the ADCs actually approved and available on commerce.

Currently, more than 20 drug conjugates are approved worldwide, of which 15 are ADCs. Research on ADCs dates back to 1980; however, the Food and Drug Administration (FDA) approved the ADCs for acute treatment only in 2000. The first-generation ADCs were derived from mice, resulting in an immune response and production of human anti-mouse antibodies. The ADC gemtuzumab ozogamicin, also known with the commercial name of Mylotarg produced by Pfizer Inc., was the first ADC approved twenty years ago by the U.S. Food and Drug Administration (FDA). Compared to first-generation ADCs, second-generation ADCs using a more toxic payload while increasing water solubility and coupling efficiency allows more payload molecules to be loaded onto each monoclonal antibody without inducing antibody aggregation. Third generation of ADCs came into being, which employ site-specific binding of small molecule drugs to monoclonal antibodies, achieving a DAR of 2 or 4 Stability and pharmacokinetic problems were greatly improved with high drug activity even at low antigen levels [10].

This review wants to summarize recent development in

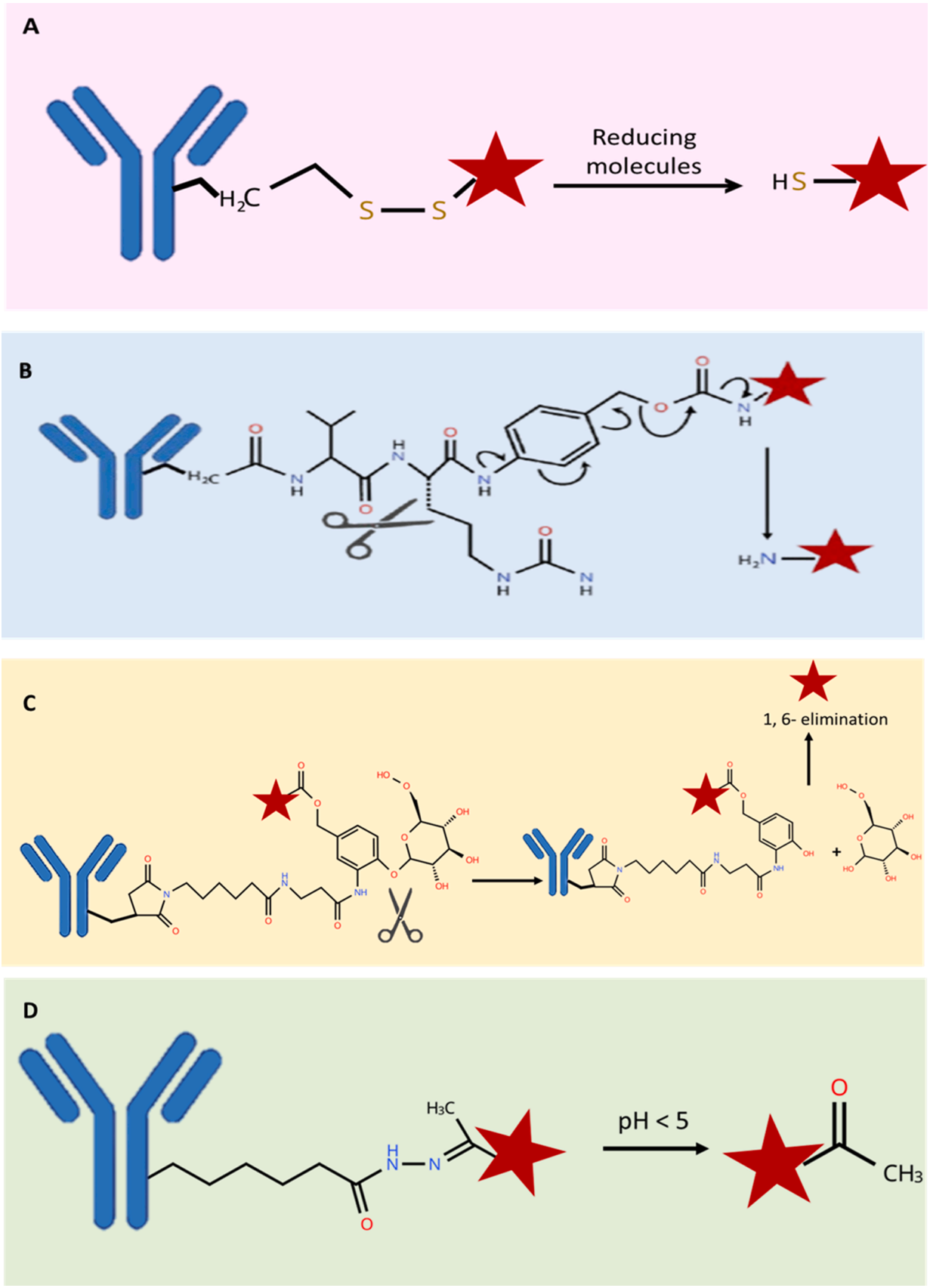

Fig. 2. chemical structure of some linkers commonly used. A: disulphide linkers, B: cathepsin B- sensible linker; C: glycosidase- sensitive linker; D: hydrazone linker.

Table 1
ADCs approved until now and some characteristics.

Time	Name	Target	mAb	Linker	Payload	DAR
2000s	Mylotarg	CD33	IgG4	Cleavable	Calicheamicin	2.5
	Adcetris	CD30	IgG1	Cleavable	MMAE	4
2010s	Kadcyla	HER2	IgG1	Non- cleavable	DM1	3.5
	Besponsa	CD22	IgG4	Cleavable	N-acetyl-γ-calicheamicin	6
	Lumoxiti	CD22	IgG1	-	PE38	-
	Polivy	CD79b	IgG1	Cleavable	MMAE	3.5
	Padcev	Nectin- 4	IgG1	Cleavable	MMAE	3.8
	Enhertu	HER2	IgG1	Cleavable	Dxd	7.8
2020s	Trodelvy	TROP2	IgG1	Cleavable	SN- 38	7.6
	Blenrep	BCMA	IgG1	Non- cleavable	MMAF	4
	Akalux	EGFR	IgG1	Non- cleavable	IRDye 700DX	1.3 – 3.8
	Zynlonta	CD19	IgG1	Non- cleavable	SG3199	2.3
	Aidixi	HER2	IgG1	Cleavable	MMAE	4
	Tivdak	TF	IgG1	Cleavable	MMAE	4
	Elahere	FRα	IgG1	Cleavable	DM4	3- 4

characterization and analysis of novel ADCs, with a special focus on capillary electrophoresis and liquid chromatography. Review on story of ADCs, biological characteristics and how ADCs work are common available on literature [21–23]. This is the reason why we want to focus more on analytical chemistry part.

2. Analytical methods for antibody drug conjugates characterization

Liquid chromatography (LC) and capillary electrophoresis (CE) are the most common analytical techniques used for physicochemical characterization of ADCs. The structural and physicochemical characterization of biopharmaceutical products requires a thorough analysis of several critical attributes. These include the full amino acids sequence, sequencing of both the N-terminal and C-terminal regions, peptide mapping, identification of free sulfhydryl groups and disulphide bonds, as well as evaluation of carbohydrate content, glycosylation profiles, glycan structures, and post-translational modifications such as oxidation and deamidation. Monoclonal antibodies (mAbs) typically possess a single N-glycosylation site on each heavy chain, located within the Fc region. Verifying the presence or absence of these glycosylation sites is a key step in mAb characterization. Furthermore, the secondary, tertiary, and quaternary structures collectively known as higher-order structures (HOS) define the three-dimensional conformation and proper folding of the biopharmaceutical molecule [24]. Any deviation from the native 3D structure may compromise protein function, potentially impairing antigen binding, increasing the exposure of immunogenic epitopes, or promoting aggregation. The biological activity and pharmacokinetics of mAbs are intrinsically linked to their ability to bind target antigens. Therefore, monitoring and characterizing this binding interaction is crucial [25]. Characterization of big molecular weight structure as ADC is a big challenge for analytical chemists.

3. Antibody drug conjugates characterization by capillary electrophoresis

CE applications for the analysis of various attributes of mAb-based therapeutic products will be examined. Capillary electrophoresis (CE) finds its main application in large molecule therapeutics analysis, where its electrophoretic separation mechanism offers a distinct and often superior separation of macromolecules compared to classic chromatographic techniques. Several modes of CE, including capillary electrophoresis sodium dodecyl sulphate (CE-SDS), capillary zone electrophoresis (CZE), and capillary isoelectric focusing (CIEF)/imaged capillary isoelectric focusing (icIEF) are commonly utilized in characterization of the critical quality attributes (CQAs) of monoclonal antibody drugs [26]. Charge heterogeneity refers to the presence of product-related variants within a biopharmaceutical that differ from the main molecule in their net charge and their presence may negatively impact the product's efficacy and pharmacokinetic profile [27]. Various capillary electrophoresis (CE) separation modes have proven effective in identifying charge variants, with capillary isoelectric focusing (cIEF) and imaged cIEF (icIEF) using ultraviolet (UV) detection being commonly implemented in quality control (QC) laboratories [28].

Zhang et al. developed an imaged capillary isoelectric focusing mass spectrometry (icIEF–MS) workflow for the characterization of cysteine-linked antibody drug conjugates (ADCs) under native conditions. Two ADCs were examined: polatuzumab vedotin, a next-generation cysteine-linked ADC, and brentuximab vedotin, the first cysteine-linked ADC approved by the FDA. This approach leverages a newly developed, MS-compatible icIEF system that enables direct coupling with a high-sensitivity mass spectrometer. Findings reveal that icIEF separation is influenced by both the drug payload and post-translational modifications (PTMs), which are subsequently identified via MS. Overall, this native icIEF–MS workflow offers valuable insights into the critical quality attributes (CQAs) necessary for ensuring the safety and efficacy of ADCs [29].

Cai et al. describes the optimization of a microchip-based electrophoresis test for analysing monoclonal antibody (mAb) product quality. This optimized method has proven suitable for platform use, as it delivers comparable performance across most mAbs in development without requiring any modifications to the procedure [30].

Belov et al employed a combined approach using middle-down and intact CZE-MS analyses to characterize a biotherapeutic monoclonal antibody (mAb) featuring a range of post-translational modifications (PTMs) and glycosylation patterns [31]. Capillary zone electrophoresis–mass spectrometry (CZE-MS) has been shown to be a viable alternative to traditional liquid chromatography mass spectrometry (LC-MS) techniques for mAb analysis [31]. Being a big challenge, various methods were validated for separation of profiling mAbs. For example, to ensure and analyze heterogeneity profile of MAbs one optimal chose could be CZE as promising platform [32,33].

In this study, Carillo et al. employed the ZipChip microfluidic CE-ESI-MS platform to analyse the drug products rituximab, trastuzumab, and bevacizumab. This technology enabled confident identification of proteoforms with an average mass accuracy of less than 15 ppm. Overall, the CE-ESI-MS platform demonstrated speed and robustness in profiling charge variants of therapeutic proteins and allowed for efficient integration with native mass spectrometry, generating highly informative characterization data [34].

François et al. present the application of an *off-line* CZE-UV/ESI-MS approach for the middle-up characterization of the Fc/2 variant of

cetuximab [35]. The results obtained from various experiments highlight the significance of this analytical method, which was specifically developed to enable a comprehensive middle-up analysis of monoclonal antibodies. One of the key advantages of this workflow lies in the implementation of an electrophoretic separation step prior to mass spectrometry. The high separation efficiency offered by capillary zone electrophoresis (CZE) proved instrumental in fully resolving different types of dimers [35]. Notably, this technique also demonstrated the ability to preserve the integrity of these dimers during analysis, further underlining its value for detailed structural characterization [35].

In this study, Giorgetti et al. developed a rapid and efficient CZE-ESI-MS method and applied it for the intact-level characterization of the commercial monoclonal antibodies trastuzumab, rituximab, and palivizumab under denaturing conditions. This approach enabled the high-resolution separation and analysis of the antibodies, providing detailed insights into their structural features and showcasing the potential of CZE-ESI-MS as a powerful tool for the comprehensive assessment of therapeutic [36]. In this study, Giorgetti et al. analyzed seven monoclonal antibodies approved by health authorities worldwide to investigate their charge heterogeneity, identify post-translational modifications (PTMs), determine their specific sites, and perform relative quantitation. The coupling of capillary electrophoresis with electrospray ionization mass spectrometry (CE-ESI-MS) proved particularly effective in detecting low-mass PTMs, providing a deeper level of structural information. The strong agreement across the analytical results highlights the reliability and efficiency of the CE-ESI-MS platform for the comprehensive characterization of monoclonal antibodies [36]. A heart-cut CZE–CZE–MS method was developed and validated by Jooß et al. for the in-depth mass spectrometric characterization of monoclonal antibody (mAb) charge variants, using a generic ε-aminocaproic acid (EACA)-based background electrolyte.

The proposed CZE–CZE–MS setup demonstrated strong potential as a powerful and versatile analytical tool for the detailed profiling of mAb charge heterogeneity, offering high resolution and enhanced structural insight [37]. In this study, has been described a very promising method dedicated to therapeutic mAbs analysis by CZE-native MS. This approach has been applied to the analysis of different therapeutic mAbs showing a great versatility [38]. Chen et al. developed a novel approach combining microfluidic capillary electrophoresis and mass spectrometry (μCE-MS) for the characterization of intact monoclonal antibodies (mAbs) and the simultaneous quantification of their variants [39]. To validate the quantitative results from mass spectrometry, a well-established capillary electrophoresis system with laser-induced fluorescence detection (CE-LIF) was employed for N-glycan profiling [40]. This study employed various capillary gel electrophoresis techniques including SDS-SGE, cIEF, and CGE-LIF to characterize a glycoengineered and a bispecific monoclonal antibody at the intact protein, subunit, and released glycan [40].

Smith et al. developed a comprehensive platform method for assessing the purity of monoclonal antibodies (mAbs) using capillary gel electrophoresis (CGE). To ensure robustness and reliability, the analytical design space was carefully defined. This included identifying critical parameters, their acceptable ranges, and optimal instrument settings. The method development process relied on a structured experimental approach, combining sequential testing with Design of Experiments (DOE) and multivariate statistical analysis to understand the influence of different variables and to optimize performance [41]. In this work, a detailed analysis was carried out to characterize the ionic and physicochemical properties of 23 monoclonal antibodies (mAbs) approved by the FDA and EMA, including representatives from the IgG1, IgG2, and IgG4 subclasses. Among these were four immuno-oncology antibodies—ipilimumab, nivolumab, pembrolizumab, and atezolizumab. The isoelectric points (pIs) of all antibodies were experimentally determined using imaged capillary isoelectric focusing (icIEF). The obtained pI values showed a high level of consistency with theoretical values predicted by bioinformatic tools such as Vector NTI and MassLynx, supporting the reliability of the experimental measurements [42]. A capillary isoelectric focusing (cIEF) method was developed and thoroughly validated for the identification testing of monoclonal antibody drug products with isoelectric points ranging from 7.0 to 9.0. The method demonstrated a high degree of reproducibility and exhibited strong specificity, not only in distinguishing between different protein therapeutics within the targeted pI range but also in detecting charge heterogeneity resulting from C-terminal lysine clipping. These features highlight the suitability of the method for both identity confirmation and detailed characterization of monoclonal antibodies [43].

An automated CIEF-MS was validated in order to separate mAb charge variants comparing with iCIEF-UV profiles. To ensure sensitivity and resolution various parameters were optimized as sheath liquid, which was one of the main impacting patterns. Additionally, glycerol was chosen as catholyte, instead the addition of Pharmalyte 3- 10 was selected as best ampholytes, reducing interferences for both CIEF and MS [44]. Dai and colleagues validated an CIEF- MS method for online MS characterization of mAb, comparing results obtained from MS with CIEF- UV analysis. The validated method was positively applied to on market available mAbs, obtaining good correlation through the two methods used. The charge variant separation was obtained using an electrokinetically pumped sheath liquid nanospray CE-MS, avoiding the ESI technology [44]. This technique has become one of the major used for characterization of mAbs and ADCs, especially for their advantages in separation of charge profile, naked mAbs and ADC, differently than other technique as ionic chromatography, due to strong interaction between stationary phase and ADC, because of their lipophilicity [45]. MS detection is the gold standard for protein identification, but its use with iCIEF is almost recent. For its approach, MS detector has overcome various problems in peak identification, adding structural information to data. The main problem is linked to type of anolyte and catholyte chosen, cause of there is the necessity to use volatile compounds (most common are ammonium hydroxide and formic acid). Glycerol is the most common coating used [46].

Hosken et al. validated a free- flow isoelectric focusing (FFE) method to isolate acidic and basic variants, previously observed through cIEF. Various individual peaks were identified from charge profile of the mAb for further identification, focusing on post- translational modification. FFE was demonstrated to be more suitable for isolating charge variants than cIEF method, obtaining excellent recovery fraction of mAb [47]. The possibility of use a gradient pH going from to 10 have permitted to separate rhumAbs with different pI. The fractionated part of the Ab was later extracted through solid phase extraction cartridge, with a recovery of more than 75 % [47]. iCIEF was used to confirm/ ensure through rapid analysis of the pI for each mAb and proteins, also in case of little difference of potential. The method was used to confirm the absence of change from the starting material after using a new chromatographic method as ion chromatography (explained in the LC part of the manuscript) [48].

Cause of the characterization of charge variants is a fundamental part of the workflow in mAbs, Wang and colleagues have performed a free-flow isoelectric focusing (FF-IEF), due to the impossibility of CEX approach. The most interesting approach was due to sample infusion flow, highly lower than normal cIEF method. This was due to the presence of hydroxymethyl cellulose in medium, and to avoid precipitation of the samples [49]. Before this newest manuscript, they have identified the structural origins of a complex charge heterogeneity of ACE2Fc, an antibody obtained through fusion between angiotensin-converting enzyme 2 (ACE2) and immunoglobulin G1 (igG1). Being heterogeneous, analysis of its charge is a challenge for analytical chemists. After optimization using hydroxypropyl methylcellulose and glycerol in water, they have separated 19 charge variant of the analyte in question [50].

Electrophoresis could be an optimal solution to characterize ADCs focusing on size variants coupled with SDS, for charge variants and pI profiling. The main advantages are directed in low sample volume and

fast methos, for major resolution mass spectrometer is suggested.

4. Antibody drug conjugates characterization by liquid chromatography

4.1. Evaluation of purity of the compound

Being these compounds useful for intravenous administration, high safety is required, especially during synthesis. First, the purity of the sample/preparation must be ensured, because preparation of an ADC require several analytical steps, with consequently many problems/ disadvantages [51]. Aggregates, fragments, particles or, more general, unwanted/undesirable parts, which could decrease the efficiency of ADC or increase toxic effects [51,52]. For example, de Mel and colleagues reported a polyphenyl RP method, with which they were able to separate the ADC by the free drug spinking the sample. They proposed three methods to quantify free drug, underling the possibility to inject direct sample without other sample preparation [53]. Jiang et al. proposed an interesting approach to identify and remove aggregates by an ADC: they used a size exclusion chromatography coupled with multi-angle light scattering (SEC-MALS) and, to control the level of aggregates ensuring the safe level of them, they characterized them by TEM [54]. Other common approaches are dynamic light scattering (DLS), which provides only presence of aggregates or pureness of the ADC, sedimentation velocity analytical ultracentrifugation (SV-AUC) or sodium dodecyl sulphate analysis (SDS), widely used in ADC's preparation to ensure batch [55].

Another common approach is using size exclusion chromatography (SEC) to purify the ADC previously synthesized. However, the main aim for SEC in this field is to quantify protein aggregates [56]. For example, Behrens et al. prepared the linker, the conjugation linker-payload and prepared the ADC using trastuzumab as Ab [57]. They used SEC to ensure the conjugation and the minimal intermolecular cross-linking of different antibody [57]. Nevertheless, SEC is not a high-resolution method, and it depends on differences of molecular weight of compounds. In Table 2 are summarized some methods used for purity of ADCs.

Different was the approach used by Khawli and colleagues, because they have chosen to use the cation exchange chromatography to separate charge variants in mAbs, later scaled- up to bigger quantities. The group have separated three different charge variants, confirming previous studies [58]. In order to separate aggregates from the pure mAb, they have chosen the SEC approach, finding major presence of aggregates in the basic region, maybe due to higher affinity for cation-exchangers [58].

Due to immunogenic response after administration of an ADC, a big challenge for analytical chemist could be the subvisible particle, regulated by USA and European Pharmacopeia. For example, Ebrahimi and colleagues have evaluated pureness of their ADC using a YMC-pack diol-200 with an aqueous mobile phase, pooling peaks before the monomer peak to quantify aggregate percentage (high molecular weight) and peaks eluted after the major peak were used to quantify fragment percentage (low molecular weight species). A similar protocol was used to avoid high- and low molecular weight species from the mAb using a TSKgel Super SW3000, 4.6×300mm column. Their conclusion has suggested that ADC with many hydrophobic payloads conjugated can cause the presence of subvisible particles [59].

As previously reported, many scientists have used SEC to separate lower and higher particles/ protein aggregates by the main product (both Ab and ADC), understanding the size by retention time. But to ensure and quantify this value approach as analytical ultracentrifugation can be used, choosing speed of rotation based on size [60]. For these reasons, Petoskey and colleagues have used light obscuration and microflow imaging or the counting and morphological characterization of subvisible particles, which were then analysed with SEC to understand the impact and quantities of aggregates [61]. These type of approach for analytical characterization of aggregates are reported in various works [62–64].

4.2. Drug antibody ratio

Drug Antibody Ratio (DAR) is one of the most important chemical characteristics of an ADC, it is linked to the efficacy and safety and represents the number of drug's molecules (payload) conjugated to the antibody. Thus, control of DAR is an important challenge for analytical chemists, because this point can result in changes in pharmacokinetics and toxicity effects [65,66]. Previously, ADCs were generated through non-specific conjugation to amino acid residues such as lysines or cysteines, resulting in heterogeneous DAR. Although antibodies have around 80 lysine residues, typically fewer than 10 are accessible for chemical conjugation due to structural and functional limitations. In the case of cysteines, the common approach involves reducing the four interchain disulphide bonds to enable drug attachment. As a result, traditional conjugation methods generally produce ADCs with between 0 and 8 drug molecules attached per antibody [65,67]. When DAR has not a well-established and repeatable value, many problems could verify aggregation, rapid clearance, drug release etc [10,68]. The conjugation of the payloads lead to become the ADC more hydrophobic, making separation a big challenge [69]. Thus, various approaches were be used to overcome these problems. In Table 3 there is a summary of the most used method for DAR quantification. Interesting was the approach used by Yolder and colleagues, which used a previous validated method LC-MS to compare two different types of conjugation, site- specific, as cysteine conjugation could be, and the lysine- linked, which is more heterogeneous conjugation. The possibility of using a site- specific conjugation gives more control in composition, analysis, and activity [70]. Despite the heterogeneous synthesis using a lysine conjugation, it represents the easiest method to obtain an ADC, thank to the high presence in Ab, surface exposure, and nucleophilicity. For example, IgG1 Ab has more than 40 modifiable lysine residues. Instead, cysteine modification is obtained through a 1- 4 conjugation to N- substituted maleimides [71].

Table 2
LC method used for ensuring pureness of ADCs.

Method	Analyte(s)	Stationary phase	Aggregates (%)	Ref.
SEC-MALS *	Gemtuzumab Ozogamicin	Superdex 200 PG resin size-exclusion chromatography	78- 44	[54]
CE-SDS-UV	26 mAb 2 ADCs	-	5< x < 80 8	[55]
SEC-MS	2 mAb 4 ADCs	BEH200 SEC 1.7 mM, 4.6 × 150 mm	0.3- 0.5 0.8- 3	[57]

* SEC- MALS: Size Exclusion Chromatography- MultiAngle Light Scattering.

Table 3
LC method for DAR quantification.

Method	Stationary phase	DAR	Ref.
HIC-MS	Tosoh TSKgel Butyl-NPR, 4.6 mm × 10 cm column, 2.5 µm particle size	≥ 1	[57]
SEC-MS	Zenix-C SEC-300 column (4.6 × 300 mm, 3 µm particle size, 300 Å pore size)	≥ 1	[57]
HIC-MS	Homemade SP	-	[77]
HIC	MabPac-Hic-Butyl column	2	[78]
RP-UV	-	2	
SEC-MALS	Acquity Premier Protein SEC, 250 Å, 1.7 µm, 4.6 × 150 mm column	3- 8	[86]
RP-UV	RP mAb Polyphenyl, 450 Å, 2.7 µm, 2.1 × 150 mm column	3- 8	
RP-MS		3- 8	
HIC-UV	BioCore HIC-Phenyl, 5 µm, 4.6 × 100 mm	3- 8	

4.2.1. UV-Visible

The simplest analytical instrument for sure is UV-Vis, which could be used to calculate DAR following some criteria, for example drug must be visible and it must have a different maximum absorbance than Ab. However, many scientists prefer to avoid this method for the possibility of interference about absorbance, for overlapping absorbance and overestimation of DAR [72,73].

4.2.2. Hydrophobic interaction chromatography

As its name suggests, Hydrophobic Interaction Chromatography (HIC) use hydrophobicity to separate compounds without losing their protein conformations. The process starts with sample injection into a high-salt mobile phase, which facilitates the binding of proteins to the surface of a moderately hydrophobic stationary phase. Often a gradient elution is used, in order to reduce salt concentration (inverse salt gradient) [74]. Usually, mobile phase A is made by 1.5-2 M of ammonium sulphate or ammonium acetate plus 0.1 M of phosphate buffer, which also represents mobile phases B. pH needs to be the most possible near to isoelectric point of the protein, instead temperature of the column must be under 30°C [75]. Ammonium sulphate is the most common buffer used for HIC, because other types (e.g., sodium chloride) can be used, but gradient needs to be arranged, stretching out retention time and total chromatographic course. Instead, buffer as ammonium acetate, maybe, needs higher molar concentration than ammonium sulphate taking other conditions equal [76]. HIC is often used prior to other analysis approach, to ensure DAR and the actual conjugation, but also in pharmaceutical industries HIC is the gold standard for quality assessment [76].

It represents the gold standard for DAR quantification, and it is important to underline that conditions used for HIC are mild and non-denaturing, preserving the native state of Ab. HIC is often used for its accuracy and rapidity to quantify ratio and DAR. However, the big amount of salt does not match with high resolution detector as mass spectrometer (MS), for this reason Chen and colleagues validated an HIC method coupled with MS using volatile salt as ammonium acetate [77]. For example, Giansanti et al. used HIC to ensure DAR, using a linear gradient with ammonium sulphate, sodium phosphate and isopropanol as mobile phase A and sodium phosphate and 20 % of isopropanol as mobile phase B [78]. The only problem with 20 % of organic solvent with high concentration of salt is the precipitation of themselves, so it is required to wash all the instrument prior and after the analysis, but, however, lower percentage of organic solvents could be better [77]. For this reason, Lovato and colleagues validated an HIC method to ensure DAR value without using organic solvent, because mobile phases were constituted by only ammonium sulphate and phosphate buffer, and with a linear gradient they were able to separate naked Ab and ADC with one and two payloads conjugated [79].

4.3. Reversed phase liquid chromatography

The most common and used type of liquid chromatography is the reversed phase, but differently than with HIC, Reversed Phase Liquid Chromatography (RPLC) strongly needs conditions to characterize an ADC, as reported in Table 3. Analysis of big molecular weight molecules like ADC can result in problems associated with strongly adsorption into stationary phase, denaturation or low sensitivity [80]. Additionally, pore size and particle diameter are variable which could influence results [81,82]. To avoid these problems some scientists decided to use very high temperatures or shorter chain stationary phases, as for example C4, or add strong acid as trifluoroacetic acid (TFA) [83].

One of the widely used approach for RPLC is proteolytic digestion or reduction by adding dithiothreitol (DTT). In this way, through various calculations based on the quantitative analysis of the drug, you can trace the calculation of the DAR [84,85]. This is what Giansanti and colleagues have done to ensure their DAR 2, using both C18 and HIC. About C18, they used 60 M excess of Tris(2-carboxyethyl) phosphine (TCEP) and analyzed drugs using a gradient method containing TFA, as previously mentioned [78].

5. Pharmacokinetics of antibody drug conjugates

Due to all of the previously mentioned characteristics of an ADC, adsorption, distribution, metabolism and elimination (ADME) have positive effects as slow clearance, long half- life, but they can have also undesirable effects if conjugation is not well carried out. For these reasons, it is mandatory to normalize pharmacokinetics (PK) profile [87–89]. There are two mainly approaches used, quantification of analytes and analysis of ADC, using high-resolution mass spectrometer.

For example, Liu and colleagues used immune- capture to take ADC from complex matrix and with cathepsin B separate the drug from the rest of the ADC and they injected into LC-MS/MS diluting sample in Acetonitrile (ACN), as best dilution solvent. This study was performed on monkeys, highlighting how changes in DAR value can affect on PK profile [90].

Yin and others validated a rapid LC-MS/MS for quantification of a free drug of their ADC, to understand PK profile, after validation of ELISA for following the entire ADC in rat and monkey plasma [66] and hybrid immunoaffinity in human plasma [67]. They used a solid liquid extraction to extract the drug working in reversed phase with MS detector through multiple reaction monitoring [68].

Lovato and colleagues validated an isocratic method for quantification of the payload and its main metabolite using HPLC-DAD. They validated the method in plasma matrix ensuring absence of interference and highlighting the possibility of using an easy and common instrumentation, as HPLC-DAD could be, to follow free drug. Quantifying both drug and metabolite is a pivotal aim in this type of analysis for future possibility of understanding PK profile [91–94]. For this reason, some times later, Lovato et al. have tried to apply their validated method to quantify the drug and its main metabolite in tissue through a liquid liquid extraction with small organic solvent volume. The performance of the method was tested on real mice samples, comparing analysis in HPLC with ELISA test, finding complementary results following two different aspect of an ADC, all the “macromolecule” with the second and the payload with the liquid chromatography [79]. The biggest goal of this research was the possibility of quantifying both drugs (main drug and its first metabolite) using a common instrumentation as HPLC- DAD, accordingly with Green Chemistry Principles.

On the contrary, Jin et al. validated a method in LC-MS following the intact ADC, extracting it through immunoaffinity from rat plasma comparing diverse DAR of the same ADC, DAR0, 2 and 8. They evaluated the presence of metabolites, based on peak percentage and retention time of them, without using standard of reference. An interesting approach that permits them to propose structural formula of metabolites [95].

There are some researches focusing on possibility of conjugated two different payloads (or more based on type of Ab) to increase cytotoxicity effects, for this reason Mak and colleagues validated a method using LC-MS to quantify six different drugs useful for ADC. They used mouse serum fortified with analyte and the extraction procedure was almost easy, because they used a liquid-liquid extraction technique with methanol and ethanol 50: 50 *v: v* for the recovery. After validation, PK parameters were obtained through collection from tail vein at different timepoints, from 0h to 8 days [96].

6. Post translational modifications (PTM)

In order to increase knowledge on site information, peptide mapping is the most used approach. PTM is a routinely analysis for whose have MS, especially in ADC’s field, to characterize modifications for mAbs and proteins, more in general [97]. For example, Wang et al. analyzed their ADC (HuN901-DM1) obtained through randomly modification of Ab, which is cross- linked to the drug in a second part. Being present six

molecules of drugs conjugated to the Ab but also naked Ab, the PTM analysis was used to identify 40 different modifications, obtaining that only 47 % of the sites are conjugated with the drug [98]. Huang and colleagues have performed a MS method with the aim of following PTM in vivo. They have focused their attention on a deamidation, quantifying both deamidated and non- form. This study has the big aim of showing what and how can happens into body after administration of an ADC, evaluating possible changes in critical quality attributes [99]. Instead, Yang and others have characterized their ADC, confirming previous results. They have employed tryptic digestion with an HPLC coupled with high resolution mass spectrometer, finding also low concentration products bio transformed and giving structural notices [100].

7. Conclusion

Due to the highly common issue of cancer and its related chemo-resistance of traditional therapies, one of the pivotal aims of recent years is to overcome these problems using targeted therapies. The interesting into ADCs approach is growing up and with it also challenges for analytical scientists to characterize, identify, and quantify this high molecular weight molecules. Someone has preferred to follow the payload (drug conjugated to Ab) other all the entire ADC. There is continuous research on this topic, trying to overcome problems related to the high molecular weight or to PK distribution. This review wanted to focus mainly on recent analytical approaches for characterization and quantification of this newest "magic bullet". For sure, there are many challenges, but various issues have already been exceeded. However, additionally, the use of green solvents and the low amount of samples represent another point to pass, trying the possibility of using more common and easier instrumentation.

CRediT authorship contribution statement

Erika Maria Ricci: Writing – review & editing, Writing – original draft, Supervision, Conceptualization. **Enrico Dainese:** Writing – review & editing, Writing – original draft, Supervision, Conceptualization. **Vincenzo De Laurenzi:** Writing – review & editing, Writing – original draft, Supervision, Conceptualization. **Giulio Lovato:** Writing – review & editing, Writing – original draft, Supervision, Conceptualization. **Gianluca Sala:** Writing – review & editing, Writing – original draft, Supervision, Conceptualization. **Marcello Locatelli:** Writing – review & editing, Writing – original draft, Supervision, Conceptualization. **Miryam Perrucci:** Writing – review & editing, Writing – original draft, Supervision, Conceptualization.

Declaration of competing interest

Authors declare no conflict of interests.

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Data availability

No data was used for the research described in the article.

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