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# Advances in native high-performance liquid chromatography and intact mass spectrometry for the characterization of biopharmaceutical products

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**Abstract**

The characterization of biotherapeutics represents a major analytical challenge. This review discusses the current state-of-the-art in analytical technologies to profile biopharma products under native conditions, i.e., the protein 3D conformation is maintained during liquid-chromatographic analysis. Native liquid-chromatographic modes that are discussed include aqueous size-exclusion chromatography, hydrophobic interaction chromatography, and ion-exchange chromatography. Infusion conditions and the possibilities and limitations to hyphenate native LC to MS are discussed. Furthermore, the applicability of native liquid-chromatography methods and intact mass-spectrometry analysis for the characterization of monoclonal antibodies and antibody-drug conjugates is discussed.

Keywords: protein therapeutics, bioprocessing, native chromatography methods, intact and native mass spectrometry, ADC, mAb.

## 1 Introduction

The demand for biopharmaceuticals, defined as pharmaceutical products originating from modern molecular biology methods, is rapidly increasing due to their successful application in the treatment of various cancers and inflammatory diseases. Currently there are more than 200 approved drugs available and the global market is expected to soon reach $278 billion [1,2]. Moreover, it is anticipated that more than 50% of new drug approvals will be biologics, rising to more than 70% by 2025 [3]. Innovations concerning the development of novel therapeutic proteins can be categorized into four groups depending on their pharmacological activity [4]. The first group involves protein therapeutics with enzymatic or regulatory activity that are prescribed to patients that exhibit protein-related deficiencies [5]. For example, a growth hormone deficiency due the lack of a specific protein that results in failure to grow at the expected rate. The second group concerns protein therapeutics with a special targeting activity. Protein therapeutics include peptides and protein derivatives [6], monoclonal antibodies (mAbs) that interact and interfere with a molecule or organism [7], and antibody-drug conjugates (ADCs) that act as a vehicle to deliver drugs to a specific biological site [8]. The third group involves protein vaccines that are used in the protection against deleterious infectious agents [9]. The fourth class regards protein diagnostic reagents that are used in clinical decision making [10].

mAbs and ADCs represent emerging classes of therapeutic agents. Over the last years more than 60 antibody derivatives have been approved by regulatory authorities for the treatment of various diseases including cancer [11], multiple sclerosis [12], rheumatoid arthritis [13], and asthma [14]. Recombinant monoclonal antibodies (~150 kDa) are composed of two identical heavy chains and two identical light chains linked by disulfide bridges, yielding a distinct Y-shape appearance. The part of the antibody which contains the antigen binding site is called the fragment of antibody binding (Fab). Large-scale production of mAbs mainly occurs

in mammalians cell cultures using host cells such as Chinese hamster ovary (CHO) cells, mouse myeloma cells such as NS0 or SP2/0 [14]. Biopharmaceutical proteins of other classes, e.g., protein therapeutics with enzymatic or regulatory activity, are mainly produced by microorganisms such as bacteria and yeasts. Mammalian cells are used for the expression of glycosylated forms of these molecules such as enzyme replacement therapies due to the requirement for specific glycan based epitopes, e.g,. mannose-6-phosphate, needed for delivery to the lysosome upon administration. Monoclonal antibodies were traditionally developed and produced using hybridoma technology, i.e., methods in which hybrid cell lines are cultivated [15]. These cells combine the ability to produce large amounts of mAbs, derived from the B lymphocytes of an immunized animal, with the immortality and high rate of reproducibility of cancer cells, derived from immortalized myeloma cells [16]. More modern approaches for the development of monoclonal antibodies include the use of techniques such as phage display and humanized mouse models for target discovery followed by molecular optimization and expression of the developed mAb using industrial scale CHO cell culture. Antibodies provide the link between the innate and adaptive immune systems thereby requiring two specific features for optimal response; 1) high and specific antigen binding as determined by the complementarity determining regions (CDRs) encoded within the variable regions of the light and heavy chains and 2) the ability to interact with Fc receptors present on innate immune cells such as macrophages and monocytes to stimulate the immune response [17,18]. Glycosylation is the biological process in which the addition of glycans or polysaccharides to the antibody takes place. Advances in protein engineering, e.g. incorporation of non-natural amino acids, have facilitated the development of mAb-related products such as site-specific antibody drug conjugates and bispecific antibodies [19,20]. Antibody-drug conjugates (ADCs) are biochemotherapeutical agents that combine the specificity of the monoclonal antibody with the cytotoxic (anti-cancer) drug [21]. ADCs are produced by conjugation of the naked monoclonal

antibody with small drugs that exert cytotoxic activity. This class of therapeutics is extremely promising in cancer treatment, and whereas some are already commercially available, e.g., Brentuximab vedotin [22] and adotrastuzumab emtansine [23], many others are under development and investigation [24]. Bispecific antibodies (bs-mAbs) can interact with two different antigens at the same time, allowing highly efficient cancer treatment. Also, composite mixtures of mAbs are being exploited as novel biopharmaceutical products. Bispecific mAbs and composite mAb mixtures enlarge the molecular complexity of drug candidates, putting even greater demands on the analytical tools to characterize them [24].

Biopharmaceuticals are much more complicated to characterize than traditional small molecule active pharmaceutical ingredients (API). Regulatory guidelines require the characterization of the primary sequence, posttranslational modifications (PTMs) and higher order structures present on these molecules, using methods such as liquid chromatography and mass spectrometry [25]. These analyses are necessary to ensure that the quality of these biopharmaceuticals is maintained and to ensure the absence of unwanted PTMs such as non-human glycosylation epitopes, e.g. galactose alpha 1-3 galactose, or the presence of aggregated forms of the drug product and sub visible particles that may be potentially immunogenic [26,27]. Chromatographic techniques such as size exclusion chromatography, cation exchange chromatography and hydrophobic interaction chromatography have, for many years, been the gold standard for the characterization of aggregates and higher order structures, charge variants and structural variants arising from PTMs such as oxidation, etc [25]. The considerable advancements in stationary phase technology, combined with the advent of high resolution mass spectrometry under native conditions represents key advances for the characterization of biopharmaceuticals [28]. As these recombinant proteins exist and exhibit their pharmacological functions as structured molecules, LC and MS methods that enable the characterization of these molecules in their native state are becoming more and more important as although still required

and powerful, bottom up approaches such as peptide mapping can often result in the loss of fine detail that exists on the molecule in its native form [29]. The ability to hyphenate native LC separation chemistries with high resolution native MS represents an emerging and important tool that will provide information that will enable the linking of sequence to structure and potential functional implications [30].

The present review aims at providing a comprehensive overview on native LC workflows and native MS strategies applied for the characterization of biopharmaceutical products. Different native LC separation modes, including aqueous size-exclusion chromatography, hydrophobic interaction chromatography, and ion-exchange chromatography are discussed. Aspects of method optimization are discussed and major applications realized with the different native LC modes are highlighted. In addition, the application possibilities of intact MS for the characterization of biopharmaceutical products are discussed and aspects of hyphenation to native LC are debated.

## 2 Native LC modes

### 2.1 Aqueous size-exclusion chromatography for the analysis of protein aggregates and fragments

In aqueous size-exclusion chromatography (SEC), biomacromolecules are separated based on their difference in hydrodynamic volume, and hence on the difference in accessibility of proteins to the intra-particle pore volume of the resin (typically varying between 35-41%) in absence of solute interactions with the stationary-phase surface. The first size-based separation of biomolecules, i.e., peptides from amino acids, was reported by Lindqvist and Storgards in 1955 using a column packed with starch [31]. The premier application area of aqueous ~~size-exclusion chromatography~~SEC with respect to the characterization of therapeutic proteins is

the quantitative determination of protein aggregation. Information on the molecular mass of monomeric proteins, possible aggregates or protein fragments is typically obtained based on a calibration curve created using protein standards. The prediction error on molar-mass accuracy provided in this way is typically around 12% (when applying a flow rate matching the minimum of the Van Deemter curve) [32]. Table I provides an overview of frequently employed SEC columns, including particle and pore size of the resins and corresponding application area with respect to the characterization of biopharmaceutical products.

Whereas the selectivity provided by the SEC column is defined by the size of the intraparticle pore diameter, the efficiency in a SEC separation is (partially) governed by the particle diameter. SEC is considered a slow and low-resolution technique, especially compared to current-state-of-the-art reversed-phase (RP-)LC columns. Due to low diffusivity of macromolecules, the optimum flow rate (corresponding to the minimum plate height in the Van Deemter curve) is very low, and in practice modern SEC columns are operated in the C-term region of the Van Deemter curve. Often columns packed with rather large particles (5 μm) are being employed, hence relatively long interparticle diffusion distances compromise the separation efficiency due to resistance to mass transfer effects. De Vos et al. discussed the need to downscale particle size to maximize resolution while exploiting the current column-pressure limitations of 20 MPa [32]. Within this pressure range it was demonstrated that SEC separations could be conducted without compromising the selectivity or altering the protein conformation by shear effects. Furthermore, it was demonstrated that a factor of 2 in analysis time could be gained when using 3 μm SEC resins instead of 5 μm particles, and optimizing the column length-to-particle-diameter ratio, such that the column efficiency was maintained [32]. The evaluation of SEC columns packed with sub-3 and sub-2-μm particles for the analysis of mAbs and ADCs was described by Fekete et al showing that an additional gain in time can be achieved without compromising analysis time [33,34]. The same group also reported the risk of forming

158 on-column aggregates when applying small-particle columns under high-pressure conditions
159 [35]. An alternative approach for method speed-up was demonstrated by Diederich et al. who
160 reported on a sub-2-minute minute method for mAb aggregate analysis using a parallel
161 interlaced SEC [36], following an approach described by Farnan et al. [37].

162 Derivatized porous silica has become the gold standard stationary-phase resin for SEC
163 columns applied to biomacromolecule analysis. To reduce strong ionic interactions induced by
164 acidic surface-silanol moieties, different surface procedures have been investigated. Diol-
165 modified silica particles have emerged as current state-of-the-art, reducing ionic interactions
166 and yielding minimal secondary hydrophobic interactions. In 2010, SEC columns packed with
167 porous hybrid organic/inorganic particulate material modified with diol chemistry became
168 commercially available, apparently reducing residual surface silanol activity, improving pH
169 stability, and increasing the mechanical strength and pressure rating of the columns [38,39]. It
170 is important to note that the adjustment of the mobile-phase pH (between pH 5.5 and 8.5) and
171 ionic strength (< 100 mM) is still required to counteract all interactions with residual silanol
172 moieties. Kopaciewicz and Regnier reported on the effects of mobile phase pH and ionic
173 strength on non-ideal protein elution behavior [40]. Applying low ionic strength mobile phases
174 (< 100 mM phosphate buffer), electrostatic interactions may affect protein retention. At salt
175 concentrations > 500 mM, hydrophobic interaction effects may occur, see also discussion in
176 'Section 2.2'. Furthermore, the extent of these interactions was determined to be protein
177 specific. Ricker and Sandoval validated these findings for the SEC analysis of mouse myeloma
178 antibodies of similar molecular weight but of varying overall charge [41]. For weakly basic
179 antibodies, good peak shapes and retention-time accuracies were observed applying mobile-
180 phase ionic strengths between 50 and 400 mM phosphate buffer pH 7. At ionic strengths > 600
181 mM peak broadening occurred and the retention time increased due to hydrophobic interactions.
182 For a strongly basic antibody an ionic strength of 200 mM was found to be optimal for the SEC

analysis, with respect to retention time and peak shape. Reducing the ionic strength led to increased retention times due to electrostatic interactions. At a concentration of 400 mM and higher, peak broadening was observed and ultimately the peak profile shifted to higher retention-time values due to hydrophobic-interaction effects affecting the size-based separation. The effect of sodium and potassium additives on protein aggregation was investigated by Goyon et al. [42]. When comparing the ratios between high-molecular species and monomers for a large number of different mAbs and ADCs, no systematic trend in aggregation level was detected. Experiments showed that the addition of sodium or potassium to the mobile phase may, to a certain extent, affect the aggregation level, but this is likely a protein-specific effect [43].

Another factor found to critically affect the SEC performance is the pH of the mobile phase, since pH affects the equilibrium between charged and uncharged forms of functional groups on both the column resin and the proteins, the latter determined by the pI, typically varying between 4 and 9 for antibodies [44]. Ricker et al. also conducted SEC experiments for mAbs applying a mobile phase with a pH-range between 7.0 and 5.5 [41]. When lowering the mobile-phase pH, protein retention increased, hence application of higher ionic-strength mobile phases was required to mediate electrostatic interactions. When applying pH 5.5 and high ionic strength mobile phases, no peak broadening or shift in retention time were witnessed. At pH 5.5 the antibody became more positively charged (shielded by the higher salt content), which makes the antibody more polar, reducing its tendency for hydrophobic interactions.

During manufacturing and storage of biopharmaceuticals, size variants can arise that can alter the safety and efficacy of the product. Although ~~size exclusion chromatography (~~SEC~~)~~ is known as the chromatographic mode with low efficiency and resolution, it is extremely powerful to assess aggregation and fragmentation. Figure 1 shows the SEC analysis of a Protein A purified monoclonal antibody recombinantly expressed in ~~Chinese Hamster Ovarian (~~CHO~~)~~

cells. This example perfectly illustrates the suitability of the technique in highlighting the presence of high and low molecular-weight variants. An important quality-control parameter that needs to be assessed during the production and storage of mAbs is the dissociation pattern of the hinge polypeptide connecting the fragment antigen binding part to the rest of the antibody. In a recent paper, Dada et al. correlated hinge fragments measured by SEC with a complementary Capillary electrophoresis – sodium dodecyl sulfate (CE-SDS) electropherogram [45]. Another important SEC application is the determination of the molecular weights of the antibody light and heavy chains. Liu et al. compared the performance of an optimized SEC methods with that of a gradient RP-LC method [46]. Whereas the retention time of the intact protein and heavy chain fragment coincided in the RP-LC methods, baseline resolution could only be achieved between intact antibody, the heavy chain and light chain fragments with SEC. A SEC method to determine the ratio of free therapeutic mAbs and anti-drug antibody complexed mAb in the serum of animals was described by Boysen et al. [47].

Hyphenation of LC, including aqueous ~~size-exclusion chromatography~~SEC, to MS detection is desired to obtain accurate mass information. Kükrer et al. described an off-line SEC-MS workflow for the analysis of dimer, trimer, and tetramer aggregates of stressed intact human monoclonal antibody (IgG) [48]. A volatile ammonium acetate buffer system yielded poor chromatographic separation and mass-spectrometry performance. To overcome this problem, monomeric and aggregate IgG fractions were collected using SEC, applying a conventional 0.1 M phosphate buffer at pH 7.2, followed by dialysis of the biomacromolecule fractions and ESI-TOF MS. Re-analysis of the dialyzed samples by SEC indicated that the oligomeric state of the different fractions was not measurable affected [48]. Shen et al. developed an on-line native SEC-MS workflow (which included a flow splitter reducing the solvent and salt intake prior to ESI) to study the effect of enzyme inhibitors on the protein quaternary structure [49]. Valliere-Douglas et al. presented a native SEC-based desalting

method for analyzing cysteinyl-linked ADCs [50]. They also studied post-desalting dissociation of the denatured ADC during ESI-ionization by comparing with an orthogonal HIC separation of the mAbs conjugated with 0-8 drugs [50].

Different SEC-ESI-MS approaches have also been developed and applied to the characterization of biotherapeutics that include the application of organic solvents in their workflow to advance the ESI spray stability [51-53]. Adding organic modifiers to the mobile phase is also frequently performed to suppress hydrophobic interactions and reduce peak tailing when analyzing highly hydrophobic biomacromolecules, such as ADCs [53,54]. It is highly probable that workflows that include organic solvents affect protein conformation, biological/enzymatic activity of biomacromolecules, protein-biomolecule interactions, and to certain extent also aggregation level. Although such workflows may be valuable, providing insights in the chemical structure, these workflows are not regarded as pure native LC. To further enhance the flow rate compatibility of SEC with MS detection and reduce the salt intake, it is mandatory to develop column technology with reduced column i.d. The number of SEC applications developed using sub 1-mm columns is limited. Rea et al. reported the use of 300 µm i.d. capillary SEC columns for mAb analysis purified from harvested cell culture fluid. After optimizing the fluidics to minimize system dispersion, picogram sensitivity was achieved in combination with UV detection [55]. Smoluch et al. applied a 300 µm i.d. column format for the on-line SEC-ESI-MS analysis of peptides in a mass range of 0.1 – 7 kDa [56].

To increase the performance of SEC, different aspects with respect to column technology and instrumentation need to be addressed. Whereas column-packing procedures for SEC columns with 5 µm particles have been fully optimized, and columns deliver reduced plate heights (h) of around 2, columns packed with small particles diameters do not yet reach their full expected kinetic performance [57]. Hence, column packing techniques to establish SEC columns need to be advanced. Also, column stability is deemed to be an issue. Recently Farrell

et al. demonstrated the long-term stability for a current state-of-the-art SEC column packed with 5 µm particles allowing for over 1500 consecutive runs, analyzing Bevacizumab aggregates, see Fig. 2 [58]. Similar experiments are required to demonstrate the robustness and the applicability of SEC columns packed with small particle diameters in a quality control environment. To further enhance the kinetic performance, core-shell particles for SEC separations may represent a good alternative to columns packed with fully-porous particles. Selectivity will be impaired, but the loss in selectivity will be small since more than 60-75% of the intraparticle pore volume is maintained. Similar to RP-LC, a gain of roughly 25% in efficiency can be expected due to improved A-, B, and C-term characteristics [59,60]. Pirok et al. demonstrated the applicability of core-shell particle technology for ~~size exclusion chromatographic~~SEC separations of polymers [61]. Columns packed with core-shell particles displayed outstanding resolution for specific (low-molecular) weight polymer separations. Furthermore, a gain in analysis speed amounting up to one order of magnitude was demonstrated.

Peak volumes provided by columns packed with sub-3-µm particles and small i.d. columns are significantly lower than obtained using conventional SEC column technology. Hence, to preserve the high efficiencies provided by these columns, it is important that the fluidic path is optimized with respect to extra-column dispersion. System-design requirements and aspect of tubing configurations influencing the separation performance have been described in a review by De Vos et al. [62]. The importance of system dispersion affecting high-resolution SEC separations has been addressed by Goyon et al. [42]. Moreover, when using small particle columns, thermal heating and possible shear-degradation effects need to be anticipated [63].

## 2.2 Hydrophobic interaction chromatography for profiling differences in surface hydrophobicity

In 1948 Shepard and Tiselius reported on hydrophobic interaction chromatography (HIC) using the term 'salting-out chromatography', observing that biomolecules bind to a hydrophobic surface material in the presence of salt [64]. Over the last years, HIC has gained significant importance for the characterization of biotherapeutics, allowing to obtain complementary information to RP-LC [65]. In contrast to RP-LC, in HIC mode, non-denaturing LC conditions are applied and hence, protein conformation and biological / enzymatic activity are maintained during the separation. When proteins are introduced in an aqueous environment, the protein surface will be shielded by ordered layers of water molecules, preventing hydrophobic interactions with the stationary phase [66]. During a HIC analysis, salt ions in the mobile phase lead to exclusion of water molecules from the surface, and the breakdown of the ordered layer is concomitant with an increase of entropy [67]. This favors the formation of hydrophobic non-covalent interactions between the hydrophobic patches situated at the proteins surface and the hydrophobic moieties on the stationary phase, decreasing the free energy. Protein elution based on difference in hydrophobic surface area is achieved by decreasing the salt concentration of the mobile phase in time.

The number of stationary phases available for HIC separations is relatively limited. This may be because effects of surface chemistry on protein conformation and hence HIC retention are still under debate. An overview of frequently used HIC columns and corresponding biopharma applications is provided in Table II. Conventional columns are packed with 5-µm diameter particles. Typically, HIC resins are less hydrophobic as compared to their counterparts used in RP-LC. The most common column material used in HIC is either surface-modified silica or polymeric particles coated with short aliphatic groups, i.e., butyl-, hexyl-, or octyl-chains [68,69]. Whereas these columns are suitable for the analysis of highly hydrophobic biomacromolecules, particles functionalized with alkylamide functionalities, polyalkylimide chemistries, and alkyl ethers are applicable for the analysis of biomolecules with a wide range

in hydrophobic surface area, including hydrophilic proteins [69]. It should be noted that different stationary-phase materials also induce protein specific retention effects [70].

HIC is typically performed applying an inverse ammonium sulfate gradient in 50-100 mM phosphate buffer pH 7. Protein retention is strongly affected by the salt concentration and the type of salt employed. The Hofmeister series, providing information on ions that stabilize the structure of proteins, has frequently been used to predict protein retention in HIC mode [71]. Soluble compounds that are well hydrated and form hydrogen bonds to water molecules will exclude water molecules from the protein and resin surface, hence promoting hydrophobic interactions. Salts that promote the formation of hydrophobic interactions are called kosmotropic, while salts that do not exhibit this property are called chaotropic [72,73]. However, Arakawa noticed that certain salts, including sodium phosphate and magnesium chloride, promote hydrophobic interactions regardless of their classification within the Hofmeister series [74]. Sodium chloride and ammonium acetate salts have been used to replace ammonium sulfate considering that the elution strength of 1 M ammonium sulfate is equivalent to ~2.6 M sodium chloride and ~3.3 M ammonium acetate [75]. Typically, kosmotropic salt systems are compatible with the analysis of hydrophilic biomacromolecules, whereas chaotropic salt systems are compatible with HIC analysis of hydrophobic proteins. To decrease retention of highly hydrophobic proteins, organic modifiers, including isopropanol and acetonitrile, are frequently added to the mobile phase [75]. The Eeltink research group recently demonstrated that the addition of only 2.5% of isopropanol to the mobile phase may lead to protein conformational changes, significantly affecting the peak profile [70]. Complementary differential scanning calorimetry analysis demonstrated that the addition of a small amount of organic modifier leads to the denaturation of the protein investigated (α-lactalbumin) [70].

Two other parameters that influence the protein retention in HIC mode are the mobile-phase pH and the column temperature. The impact of pH depends on the isoelectric point of the

protein. Good practice is to minimize the shift in pH between the pI of the protein and the pH of the mobile phase, to prevent possible 3D conformation changes, affecting the level of protein aggregation, or even induce protein denaturation. The effect of temperature on HIC separations is still under investigation [76,77]. Generally, it can be affirmed that an increase of temperature (i.e. column temperature) drives an increase in protein retention. The formation of hydrophobic interactions is an entropy driven process and the temperature increase favors a decrease in free energy. On the other hand, the increase of (column) temperature can induce undesired conformational changes of proteins, and possibly lead to a change in the strength of the hydrophobic interaction when the surface hydrophobicity is altered [78]. A safe range is retained to be in the temperature interval between 20°C and 40°C [79].

HIC has been successfully applied to characterize mAbs with respect to profiling post-translational modifications, including monitoring of oxidation variants [80], aspartic-acid isomerization [81], and domain misfolding [82]. In particular, oxidation of the amino-acids exposed to the storage environment and micro-heterogeneities in the carboxy terminal chains are common post-translational modifications that need to be monitored in order to guarantee the quality of mAbs products. Boyd et al. described the separation of native IgG1 from its oxidized Trp counterpart [80]. The authors also claimed that the HIC approach allows for profiling of oxidized methionine and isomerization/deamidation products. A comprehensive study to characterize mAbs variants resulting from variable N- and C-terminal processing and stress-induced modifications using HIC technology was performed by Valliere-Douglass et al. [81]. In this study, the authors also demonstrated the applicability of HIC to separate truncated antibodies from native species.

One of the key HIC applications is the determination of the average load of cytotoxic drug with respect to the antibody, i.e., the average drug-to-antibody ratio (DAR) of ADCs. Having information of the average DAR ratio is essential, since this value determines the

quantity of cytotoxic drug that will be transported to the targeted tumor cell, defining the efficacy of the chemotherapeutical distribution. Fig. 3 shows the HIC separation of ADCs having different payloads [82]. The peaks were assigned using the unmodified antibody for the zero-drug peak and the absorbance ratio measured at 248 and 280 nm for the other peaks, since the drug and antibody have distinct absorbance maxima at these wavelengths. The cytotoxic drugs applied are typically hydrophobic, hence when the payload increases also the HIC retention time increases and the DAR ratio can be calculated by summation of the individual peak areas multiplied with their respective drug load divided by the total peaks area. Depending on the type of mAb (IgG1 or IgG2) used, the DAR varied between 2 and 8 for IgG1 and between DAR 2 up to 12 for IgG2 [83]. DAR 0 refers to the mAb in which the conjugation with the cytotoxic drug did not occur, while odd DAR numbers (normally present in neglectable amounts) refer to ADC in which the conjugation is I ncomplete. The latter two cases are considered as impurities in ADC analysis. In the case of ADCs derived from IgG1, different positional isomers can be present in the DAR 2, DAR 4 and DAR 6 forms. Unfortunately, HIC has no sensitivity towards positional isomers whereas capillary electrophoresis-SDS-PAGE [84] and also ion mobility may well have. The characterization of ADCs and their payloads using comprehensive LC modes has been described in an excellent review by Bobaly et al. [85].

## 2.3 Ion-exchange chromatography for the analysis of charge variants

The relevance of ion-exchange chromatography (IEX) in biochemical studies was demonstrated already in 1949 by Cohn, who performed cation and anion-exchange separations for a trace-analysis study on the enzymatic formation and degradation of nucleic acids [86]. In recent years, IEX has been widely applied to monitor product quality and consistency of biotherapeutics. The separation is based on coulombic interactions between the stationary-phase surface, containing ionic functional groups, and the charges of the therapeutic protein.

Since the disposition of charges at the protein surface depends on the native 3D protein conformation, proteins having structural diversities can be differentiated by means of IEX. The net charge of a therapeutic protein is not only determined by the amino-acid residues on the protein backbone, but also charged glycans are accounting for a portion of the net charge of the protein. These charges not only affect the structure of the protein, and thus determine the stability and solubility of the therapeutic product, the charges also affect the binding affinity to receptors and functional groups of the stationary phase, influencing its biological activity [87,88]. The versatility of IEX in protein analysis is related to the fact that a wide range of separation conditions with respect to salt concentrations and pH are applicable. An overview of frequently used columns for the IEX characterization of biotherapeutics is provided in Table III. The maximum pressure rating of the current commercially-available material is currently 40 MPa, limiting the application of IEX under UHPLC conditions and thus also its possibility for method speed-up. The majority of applications is performed using 4.6 or 2.1 mm i.d. column formats. Rea and Farnan reported on the use of capillary columns formats, i.e., 400 µm i.d. columns packed with 5 µm pellicular strong cation-exchange particles and 300 µm i.d. columns packed with 1.7 µm non-porous weak-cation exchange particles for the separation of mAb charge variants [89].

IEX separations can be performed using a salt gradient while keeping the mobile phase pH constant. This increase in ionic strength of the mobile phase promotes protein elution as the salt ions compete with the adsorbed protein molecules for the ion-exchange sites on the resin. Salt gradients provide good resolving power and robustness, but are product specific and time-consuming to develop. Sodium chloride, usually dissolved in a < 50 mM sodium phosphate buffer, is the most-employed eluent for separating proteins using salt gradients [90-92]. It is assumed that NaCl does not affect protein conformation. As the nature of the buffer cation and anion can affect protein retention and peak widths, the selection of the ideal salt buffer system

is very important. [93,94]. The effects of eluent salts on the resolution of protein separation has been described by Gooding et al., and Regnier et al. [95,96]. Not all charge variants are generally resolved using a salt gradient in IEX mode, especially the acidic variants [97]. As the pH is remained constant during the elution process, proteins with the same effective charge will be eluting with poor resolution. Nevertheless, the potential of CEX for mAb characterization, applying a shallow gradient of increasing salt concentration (typically 200 mM NaCl) at constant pH, has been reported in several publications [90,98]. Flattening of the salt gradient only improves the resolution if the pH of the separation is operated near the pI of the proteins to be analyzed. As it is demanding in a high-throughput quality control environment of biopharmaceutical industry to tailor salt systems for individual mAbs, alternative elution approaches are preferred.

Proteins can also be eluted in IEX mode by generating a pH gradient across the column. Irrespectively of how the pH gradient is formed, two modes of chromatofocusing can be distinguished, i.e., cation chromatofocusing where the stationary phase exhibits cation-exchange properties and a gradient running from low to high pH is generated, and anion chromatofocusing which employs an anion-exchange resin and proteins are eluted by applying pH gradient going from high to low pH. Generating pH gradients in IEX mode is generally called chromatofocusing, which is a pressure-driven chromatographic variant of isoelectric focusing (IEF) elution mechanism coined by Sluyterman and Elgersma [99-101]. Whereas conventional chromatofocusing using an "internally-generated" pH gradient, gradient chromatofocusing employs an "externally-generated" pH gradient. In the former variant of this separation method, the buffer capacity of the stationary phase is used to convert a step change in pH after applying a mobile-phase of a given pH at the column inlet, while the IEX resin is pre-adjusted at a different initial pH. In this way, an internally-generated pH gradient is generated as the packing material will buffer the pH step. This travelling pH wave allows to

focus proteins, and releasing them once the pH gradient approaches the pI of the biomacromolecule. To generate an internal pH-wave, either an immobilized ampholytic buffer bound to a strong ion exchange resin, or non-interacting buffer species in conjunction with a weak ion-exchange resin being used [102-104]. The major challenge is to precisely generate the required pH gradient, while minimizing the ionic strength of the running buffer to reduce its effect on protein retention. The conventional elution buffers are polyampholytes. These molecules provide a high buffer capacity covering a broad pH range, but are poorly defined, and have been reported to interact with both the proteins as with the stationary phase resin [105]. Alternatively, a combination of equally concentrated buffer species with equally spaced pKa values in the chosen pH range can be employed. Kröner et al. provided an in-silico optimization method of buffer compositions, resulting in well-controllable pH gradients with low ionic strength validated for characterization of more than 20 proteins [106].

Alternatively, chromatofocusing can also be performed by applying an externally-generated pH gradient, i.e., by use of the gradient proportioning system of the LC pump. By gradually mixing the running buffer with successively greater proportions of an application buffer, while both buffers are set at different pH, a pH gradient is generated in time prior to entering the column. At the start of the pH gradient proteins are adsorbed on the column head and the proteins elute once the incoming pH gradient is slightly below the pI of the protein. The quality of the separation is thus depending strongly on the solvent-proportioning capabilities of the LC equipment, as poorly controlled pH gradients can result in co-elution of proteins with similar pI's. The formation of multi-step or multi-variable slope (non)-linear gradients over a wide pH range, and a buffer system compatible with both anion- and cation-exchange stationary phases that allows for an arbitrary start- and end-pH value and pH-range are still not available for this separation mode [107]. Tsonev and Hirsch developed software that can precisely perform high-order polynomial fitting of titration curves for a dedicated buffer system, allowing

controlled gradient formation of any desired shape and slope [107,108] for both cation- and anion-exchange separations. Furthermore, the algorithm also allows for software-driven control of pH gradients that can contains additives such as nonionic detergents, organic modifiers, salts, etc. Fig. 4 shows the comparison between the optimization of an anion exchange separation of E. coli acetone powders performed using a salt gradient (Fig. 4A), and using a pH gradient (Fig. 4B). The steepness of the salt gradients was decreased, at the expense of analysis time, whereas for the pH gradient separations only the slope of the gradient between pH 3.5 and 2.4 was varied, see Fig. 4C. The pH gradient separations are offering the best resolution, especially for the very acidic proteins present in the complex E. coli mixture.

IEX has emerged as the standard method for the determination of charge heterogeneity of monoclonal antibodies. It is important to measure product heterogeneity during the development and production process of mAbs, as many charge variants can arise due to post-translational modification or product degradation processes. These modifications processes of the parent protein include C-terminal lysine variants, N-terminal pyroglutamate formation, deamidation, glycation, and glycosylation, resulting in a modified isoelectric pH (pI) value of the mAb [109,110]. Vlasak et al. has written a review on the analysis of charge-related heterogeneity in monoclonal antibodies [111]. IEX is less preferred to study ADC charge variants, as the linked cytotoxic drugs are changing the hydrophobic surface of the conjugated antibody resulting in unwanted secondary interactions with the stationary phase and consequently a poor resolution separation [112]. Some studies have been performed on retention time models for IEX separations using salt gradients [113,114] and pH gradients [115]. Fekete et al. applied a Drylab CEX model for the separation of mAb charge variants using both salt and pH gradients [116,117].

The contribution of various posttranslational modifications to monoclonal antibodies is diverse, with basic amino acids contributing to an increase in the mAbs pI whilst deamidation

of asparagine residues and sialic acid present on N-glycans contribute to a decrease in the mAbs pI. These different contributors to the overall protein chemistry of the mAb make cation exchange (CEX) chromatography the analysis method-of-choice to study mAb charge variants. In CEX mode, the separation of proteins is governed by the surface-charge, charge-distribution, and the geometry of the protein. CEX separations of mAbs is typically performed by applying a gradient with increasing salt concentration (i.e. 100-200 mM sodium choride), while maintaining the pH of the buffer constant. The pH of the buffer depends on the isoelectric point of the mAbs under analysis but in general the pH range is between 7.5 and 9 [118]. Separation of mAbs in anion exchange (AEX) mode, is also being performed, mainly to separate oxidized variants of mAbs. Teshima et al. showed how AEX was effective in the analysis of three force-oxidized antibodies as compared to CEX. It was demonstrated that AEX revealed oxidized mAbs variants not monitored using CEX [119].

Jungbauer demonstrated in 1993 the combined effect of a linear salt- and pH-gradient in IEX mode for the separation of protein isoforms of a human monoclonal antibody [120]. The IEX chromatogram was compared with IEF, and confirmation of elution order based on pI was shown. As the method relied on the reaction of mannitol with borate, the broad-scale applicative value was limited. Many publications have investigated internally generated pH-gradient CEX methods to separate mAb charge variants, however, they often employ cationic buffering agents which can lead to interactions with the stationary-phase chemistry. This deviates the shape of the applied pH-gradient from the ideal linear case, affecting protein retention and the resolution of the separation [121-123]. In an attempt to address this issue, research groups have investigated algorithms to correct for these deviations [107], a simple mixture of buffering species which produce an internal linear gradient for neutral and acidic mAbs [124], mixed-bed stationary phases consisting of small-pore weak IEX and large-pore strong IEX particles allowing for independent internal pH-gradient generation and protein binding [125,126],

external pH gradients in anion-exchange mode using a mixture of amine buffering species as an application buffer and weakly acidic compounds as an elution buffer [127-129], shallow externally generated pH gradient of diethanolamine buffer on monolithic IEX stationary phases [130,131].

Another way of solving this issue is by using zwitterionic and acidic buffer substances with a pKa-range evenly distributed over the pH range and externally generate a pH and salt gradient. Typically, zwitterionic compounds tailored for biochemical research are used as buffering agents. It was shown that this allows for generating highly linear pH gradients, with even distribution of buffer capacity, for the analysis of charge heterogeneity of mAbs [132]. Recently the ruggedness of a controlled gradient pH-formation with a zwitterionic buffer system for the separation of mAb charge variants was demonstrated, showing good robustness of the method with < 0.8% RSD for the retention times after more than 300 injections [133].

## 3 Protein mass spectrometry

Mass spectrometry (MS) for intact protein analysis has proven to be essential in the field of biomolecule characterization. Fenn received the Nobel Prize in Chemistry in 2002 for the development of electrospray ionization (ESI), allowing the transfer of biomacromolecules from an aqueous solution into the gas-phase as molecular ions without fragmentation [134,135]. ESI can operate in the flow regime from 1 mL/min. down to tens of nL/min., with the latter approach typically utilizing “static” (i.e., off-line) spray from glass capillaries, also called nano-ESI [136]. Electrospray is now the dominant ionization method in many chemical, (bio-)medical and pharmaceutical mass spectrometry laboratories, largely due to the ease with which it can be used to interface LC “in-line” with different types of MS(/MS) instruments [137]. Denaturing MS-based strategies have been extensively applied to retrieve information on the

intact mass of therapeutic proteins, including information on the amino-acid sequence and post-translational modifications, the DAR and drug load distribution, etc [138].

A visualization of the different states of protein MS analysis is depicted in Fig. 5. Proteins encountered at physiological conditions remain their native 3D structure. In "intact denatured proteins" the 3D protein conformation is lost. The protein mass is particularly important when variations of the amino-acid sequence, such as mutations or truncations, as well as post-translational or chemical modifications, e.g., deamidation, covalent linkers, need to be identified and quantified [138]. While the full set of modifications present as well as heterogeneity arising from the occupancy of the possible sites can be obtained from intact, denaturing MS, mapping the modification sites requires MS/MS approaches typically using bottom-up proteomics, but increasingly also middle-down and top-down MS/MS [139]. The often overlooked, but important shortcoming of these "standard" proteomics methods however is that they usually only give partial sequence information (i.e. not all expected peptides or MS/MS fragments are found back in the spectra), and therefore neither identify all modification sites nor typically characterize the full complement of modifications, i.e., their extent and heterogeneity). It has become increasingly obvious that the full knowledge of the primary sequence information, the "proteoform", i.e., the "chemical sum formula" of the protein and its sequence) [140], can only be obtained by a combination of intact protein MS with MS/MS approaches as they are used in proteomics, with or without prior digestion.

Extending the applicability of ESI-MS incorporating volatile buffer systems and physiological pH conditions, as well as modifications to the instruments to increase the mass range and the control over desolvation conditions, has led to the development of native MS [141,142]. Native MS has been extensively applied to study macromolecular assemblies, including stoichiometry and identity of binding partners [143,144], and in the last decade its applicability has been extended towards the MS analysis of biopharmaceutical products [145].

In native MS, it is believed that non-covalent weak interactions, i.e., van der Waals interactions, hydrogen bonds, electrostatic interactions, are maintained, preserving the higher-order, three-dimensional protein structure during the MS analysis. It is generally recognized that changes in charge density in ESI-MS spectra correspond to conformational changes, i.e., the tertiary protein structure [146]. Fig. 6A displays the charge-state distribution profile of an intact protein (anti-thrombin III) applying native MS conditions [147]. Due to the compact, folded state of the protein, the exposed surface that can be protonated is relatively small, therefore yielding a relatively narrow charge envelope situated in the high m/z region (low z) compared to the same protein when applying denaturing ESI-MS conditions (Fig. 6C). Fig. 6B shows that native and denatured protein states coexist at equilibrium applying mildly denaturing conditions. The intermediate charge-density ions correspond to proteins that contain domains that are unfolded, while other domains retain their native conformation. While unfolding proteins in denaturing MS usually allows accurate and precise mass determination (≤ 1 Da), desolvation conditions are more gentle in native MS and the folded protein often retains bound water or buffer ions, leading to a somewhat increased experimental mass compared to the expected value [148]. When determining the intact mass of proteins above 100 kDa on the other hand, the native approach may become easier, as it produces fewer and lower charge states, whereas denaturing MS leads to a large number of closely spaced, highly charged peaks which are difficult to resolve and to correctly assign.

Ion mobility (IM) is now often coupled with native MS and several commercial platforms offer this option [142]. In ion mobility, ions are separated by their collision cross section (CCS), measured in $nm^2$ or $Å^2$, which depends on their charge but also their rotationally averaged size and shape – somewhat similar to gas-phase electrophoresis [149]. At each m/z, different co-existing conformers, isomers or complex/aggregate topologies can be resolved as long as they differ in overall size by 2-3%. The measured mobilities of ions can be converted

582 to CCS values using a set of calibrants (e.g. protein standards) with known structure. This
583 technology has come to the fore in the last 10 years and recent examples include studies of
584 protein folding/misfolding and aggregation, intrinsic disorder phenomena and the identification
585 of isomeric forms of metabolites, biomolecules and complexes [150]. In the context of
586 biopharmaceuticals characterization, ion mobility has shown to be able to distinguish different
587 glycoforms, even in cases where they cannot be resolved in LC, as well as disulfide isoforms
588 [151].

589

590 **3.1 Conditions for direct infusion and hyphenation to LC**

591 Gentle ionization, in which the non-covalent interactions involved in protein higher-
592 order structure, i.e., folding and interactions, are maintained, is considered to be a critical step
593 in native MS [146]. Most native MS is done in "static", off-line nano-electrospray ionization
594 (nano-ESI) using metallized glass capillaries, also called direct infusion, with a flow rate < 20
595 nL/min in order to minimize sample consumption, improve the tolerance of spraying aqueous
596 buffer solutions, limiting the salt intake, and eliminating the need for desolvation gas and
597 heating. Native MS can also be implemented at flow rates in the 200-300 nL/min. range which
598 are compatible with in-line nano-LC, and in principle also at higher flow rates, although care
599 has to be taken that ESI interface settings such as (hot) desolvation gas and source heating do
600 not unfold the protein. Sample requirements for native MS and buffer conditions have been
601 described by Hernandez and Robinson [152]. Typically, infusion of analyte at 1-20 μM
602 dissolved in 10 mM – 1 M aqueous ammonium acetate solution maintained near pH 7 or at the
603 pH of choice, using an excess of ammonia or acetic acid, provides good MS spectra. Also, other
604 ammonium salts and ammonium derivatives have been employed but acetates are found to
605 perform better than bicarbonates [153]. Non-volatile ions such as sodium and potassium are
606 minimized using buffer exchange and other desalting methods, since these salts induce adduct

formation, thereby lowering the mass resolution or suppressing signal entirely. Essential co-factors such as e.g. Mg or Zn ions can be added, but a large excess should be avoided. With respect to optimization of the MS settings, it is important that pressure in the transfer region between source and analyzer is optimized to ensure transmission of biomacromolecules. Modifications of MS instrumentation has been described in more detail by Rosati et al. [151].

Due to the stringent requirements with respect to infusion conditions, the number of reports describing the direct coupling between native LC and mass spectrometry is limited. First of all, the flow rate compatibility with LC constitutes a problem. Conventional SEC, HIC, and IEX separations are still performed using either 4.6 mm i.d. columns operated at a flow rate of 1 mL/min, or 2.1 mm i.d. columns operated at 0.2 mL/min. Hence, post-column flow splitting is required to achieve direct coupling to MS via nano-ESI. Furthermore, the salts typically applied in SEC, HIC and IEX modes are incompatible with MS analysis. In case of aqueous SEC, the phosphate buffer can be replaced by an acetate buffer. In gel filtration, typical buffers are fully native, but scaling down is a major bottleneck. The sulfate ions typically used in HIC systems are also incompatible with MS, leading to significant signal suppression [154]. Volatile buffer acetate and tartrate and ammonium salt systems can be considered for HIC-MS analysis, b~~i~~ut it should be noted that the choice affects protein retention and may limit the applicability. Xiu et al. reported a lack of retention for the HIC analysis of hydrophilic proteins using ammonium acetate as the mobile phase (as can be expected from the Hofmeister series) [154]. Ammonium tartrate dissolved in an ammonium acetate buffer provided similar elution strength compared to ammonium sulfate. The MS compatibility with respect to adduct formation was only demonstrated after desalting using ultra-centrifugation followed by RP-LC-MS analysis. It has been reported by Chen et al. that the desalting processes can induce variations in the conformation of the proteins, and hence the native conformation may be lost [155]. A viable

approach to online HIC-ESI-MS was proposed by Chen et al., which involves the use of low concentrations of ammonium acetate mobile phases (volatile and MS compatible) [155].

### 3.2 MS characterization of mAbs and ADCs: key examples

A protocol for conducting native MS analysis of mAbs and ADCs has been described by Thompson et al. [146]. Illustrative MS spectra of a 145 kDa purified mAb via direct infusion are demonstrated in Fig. 7A, yielding only seven charge states. A mass accuracy of 5 Da allows establishing the protein i.d. with high confidence when the amino-acid sequence is known and allows identifying modifications, including primary sequence mutations and C-terminal lysine clipping [146]. The natural isotopic peak width of the intact antibody was estimated to be 25 Da. A very accurate isotope pattern needs to be recorded in order ~~Hence, the mass resolution currently achieved using time-of-flight instruments is too low~~ to detect modifications such as deamidation, yielding a mass increase of +1 Da. Significant improvements in resolution and native MS technology have been reported over the years. For example, in 2012, Rose et al. reported the use of Orbitrap MS yielding a resolution of 16,000 at m/z 10,000 [156]. The applicability of native MS to probe the binding stoichiometries and affinities of mAb-antigen complexes was first demonstrated by Tito et al. [~~ref~~157]. Compared to SEC/UV or SPR spectroscopy yielding evidence for binding, or at best average-weight information, native MS provides accurate mass information. Tito et al. also performed control experiments to establish the specificity of the interactions [157].

The presence of microheterogeneities in the protein chains can derive from inconsistencies in the production process (differences in the cell lines) and therefore it is extremely important to perform batch to batch quality control of the mAbs before their application as therapeutics. Most of the times these inconsistencies stem from heterogeneous glycosylation patterns. Fig. 7B shows native MS spectra of a mAb with glycosylation and after

deglycosylation using peptide-N-glycosidase F [146]. The presence of glycans increases both the mass and the heterogeneity of the MS signal, that in turns decreases the peak intensity. Intact MS analysis can be used to reveal the presence of different glycoforms or on the chains of the mAbs (mutations on the mAbs chain can dramatically alter the glycosylation) [ref]. Rosati et al. performed both qualitative and quantitative analysis of glycosylation profiles on mAbs using high-resolution Orbitrap MS technology [151]. Fig. 8 compares native MS spectra of IgGs obtained via direct-infusion experiment and ~~that obtained~~ after on-line SEC analysis, as performed by Chatterjee and Sobott. This experiment showed that the SEC analysis induced partial~~y~~ unfolding of the antibodies (without breaking disulfide bonds), as it appears with higher charge states.

With respect to the analysis of ADCs, MS enables the characterization of the drug load profile and distribution, and the DAR. Valliere-Douglas et al. and Sobott et al. reported on a method allowing to determine the intact mass of an ADC composed of non-covalently-associated heavy and light chains, with a drug linked to interchain cysteine residues [158, 159]. Debaene et al. conducted native MS experiments of Brentuximab vedotin (also an inter-chain cysteinyl-linked ADC) providing accurate mass measurements of intact ADCs together with the average DAR and drug distribution [160]. The same group also characterized a lysine linked antibody drug conjugate (Tratuzumab emtansine) [160]. Extending the glycoprofiling experiments of mAbs, Rosati et al. also characterized the drug load and glycosylation patterns on an IgG4 ADCs using high-resolution native MS [151].

## 4 Concluding remarks

Advances in the development of biotherapeutics are closely followed by innovations in the field of separation sciences and mass spectrometry. The chemical heterogeneity of biopharmaceuticals in terms of polarity, size, and charge, require the use of complementary

native LC techniques ideally hyphenated to mass spectrometry to fully characterize (and quantify) the complex protein samples. This requires the use of separation technology with high resolving power to achieve the highest confidence in elucidating the biopharmaceutical product. Although columns packed with sub-2-μm particles are being introduced and have become commercially available, the majority of LC experiments is still performed using conventional columns packed with 5 μm particles. However, to make a successful transition the effects on protein unfolding induced by the mobile-phase composition applied, shear stress, and thermal effects, need to be critically assessed. Furthermore, the pressure stability of currently available IEX, SEC, and HIC columns needs to be augmented in order to allow for operating pressures above 50 MPa. A promising (but currently underestimated) stationary-phase type for biomacromolecule separation may be monolithic columns. The morphology can be optimized to achieve high efficiency separations by down-scaling the globule size, while the macropore size can be tuned to minimize shear stress.

Conventional 4.6 mm i.d. analytical columns for mAb analysis, require several micrograms of mAbs to achieve adequate detection sensitivity of low-abundant sample species. The yield of biopharmaceutical products coming from microwell-plate cell cultures is however limited and often insufficient for high-resolution LC analysis. This mandates the miniaturization of column formats allowing to increase detection sensitivity and to diminish sample consumption. It should be noted however, that extra-column band broadening needs to be minimized, imposing stringent requirements on instrumentation. An additional advantage of reducing the column format, is that it decreases the salt-intake at the MS interface, effectively improving MS compatibility. The use of organic solvents that are conventionally added to the mobile-phase, not only to improve spray drying but also to reduce the surface tension of the spray droplets leading to higher ionization yields, should be limited when performing bioanalysis. This is not only mandatory to maintain the protein conformation but also because

706 buffers are known to lead to suppression of ion formation in the ion source and ion source
707 contamination due to salt crust formation. Novel salt systems, buffering agents, the effects of
708 ionic strength have to be further studied in order to improve MS compatibility. At the same
709 time, it is mandatory to further study the effects of ionization conditions and MS conditions on
710 protein conformation, in order to establish relevant biological conditions.

711

## List of abbreviations

ADC Antibody drug conjugate

CCS Collision cross section

CDRs Complementarity determining regions

CHO Chines Hamster Ovary

DAR Drug to antibody ratio

E. Coli Escherichia Coli

HIC Hydrophobic interaction chromatography

IgG Immunoglobulin G

mAb Monoclonal antibody

PTM Post translational modification

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**Table I.** Overview of SEC columns applied for the separation therapeutic proteins frequently reported in scientific literature.

| Column | Matrix | Chemistry | Particle size (µm) | Pore size (Å) | pH stability | Max Pressure (MPa) | Brand |
|---|---|---|---|---|---|---|---|
| Advanced Bio SEC | Silica | Silanol | 2.7 | 300 | 2-8.5 | 10 | Agilent Technologies |
| Yarra SEC-X300 | Silica | Silanol | 1.8 | 300 | 1.5-8.5 | 48 | Phenomenex |
| Unix-C SEC 300 | Silica | Diol | 1.8 | 300 | 2-8.5 | 31 | Sepax Technologies |
| Zenix SEC-300 | Silica | Diol | 3 | 300 | 2-8.5 | 7 | Sepax Technologies |
| MAbPAc SEC 1 | Silica | Diol | 5 | 300 | 2-7.5 | 7 | Thermo Fisher Scientific |
| TSKgel SuperSW mAb HR | Silica | Diol | 4 | 250 | 2-7.5 | 8 | Tosoh Bioscience |
| TSKgel UltraSW Aggregate | Silica | Diol | 3 | 3000 | 2-7.5 | 12 | Tosoh Bioscience |
| Protein-Pak SEC | Silica | Diol | 10 | 300 | 2-8 | 30 | Waters |
| XBridge Protein BEH SEC | Silica | Silanol | 3.5 | 200 | 1-8 | Not available | Waters |

**Table II.** Overview of HIC columns applied for the separation therapeutic proteins frequently reported in scientific literature.

| Column | Matrix | Chemistry | Particle size (µm) | Pore size (Å) | pH stability | Max Pressure (MPa) | Brand |
|---|---|---|---|---|---|---|---|
| Proteomix HIC 1.7 | PS/DVB | Butyl/Ethyl | 1.7 | Non porous | 2-12 | 50 | Sepax Technologies |
| Proteomix HIC 5 | PS/DVB | Phenyl/Butyl/ Propyl/Ethyl | 5 | Non porous | 2-12 | 41 | Sepax Technologies |
| MAbPAc HIC-10 | Silica | Alkyl amide | 5 | 1000 | 2-8 | 55 | Thermo Fisher Scientific |
| MAbPAc HIC-20 | Silica | Alkyl amide | 5 | 1000 | 2-9 | 55 | Thermo Fisher Scientific |
| MAbPAc HIC-Butyl | Polymer | Poly amide | 5 | Non porous | 2-12 | 27 | Thermo Fisher Scientific |
| TSKgel Butyl-NPR | Polymethacrylate | Butyl | 2.5 | Non porous | 2-12 | 20 | Tosoh Bioscience |
| TSKgel Phenyl-5PW | Polymethacrylate | Ether | 13, 10 | 1000 | 2-12 | 2 | Tosoh Bioscience |
| TSKgel Ether-5PW | Polymethacrylate | Polyamine | 10 | 1000 | 2-12 | 2 | Tosoh Bioscience |
| Protein-Pak Hi Res HIC | Polymethacrylate | Ether | 10 | Non porous | 2-12 | 20 | Waters |

**Table III.** Overview of IEX columns applied for the separation therapeutic proteins frequently reported in scientific literature.

| Column | Matrix | Chemistry | Particle size (µm) | Pore size (Å) | pH stability | Max Pressure (MPa) | Brand |
|---|---|---|---|---|---|---|---|
| Agilent Bio SCX | PS/DVB | Sulfonic acid | 10, 5, 3, 1.7 | Non porous | 2-12 | 68 | Agilent Technologies |
| WP CBX | Silica | Sulfonic acid | 5 | 300 | 2-8 | 45 | Avantor Inc |
| Antibodix WCX | PS/DVB | Carboxylate | 10, 5, 3, 1.7 | Non porous | 2-12 | 68 | Sepax Technologies |
| Proteomix SCX | PS/DVB | Sulfonic acid | 10, 5, 3, 1.7 | Non porous | 2-12 | 68 | Sepax Technologies |
| BioBasic SCX LC | Silica | Sulfonic acid | 5 | 300 | 2-8 | 40 | Thermo Fisher Scientific |
| MabPac SCX | Polymer | Sulfonic acid | 10 | Non porous | 2-12 | 20 | Thermo Fisher Scientific |
| TSKgel Q-STAT | Polymer | Quaternary ammonium | 10 | Non porous | 3-10 | 5 | Tosoh Bioscience |
| TSKgel Bioassist Q | Polymethacrylate | Polyamine | 13, 10 | 4000 | 2-12 | 2 | Tosoh Bioscience |
| Protein-Pak HiRes CM | Polymethacrylate | Carboxymethyl | 7 | Non porous | 3-10 | 15 | Waters |

**Figure captions**

**Figure 1.** SEC analysis of a Protein A purified monoclonal antibody recombinantly expressed in Chinese Hamster Ovarian (CHO) cells performed on a 7.84.0 mm i.d. × 300 mm long AdvanceBio SEC column packed with 2.7 μm particles containing 300Å pores. Separation conducted applying a mobile phase of 150 mM sodium phosphate pH 7, a flow rate of 0.8 mL/min, and UV detection at 220 nm.

**Figure 2.** Overlay of selected SEC chromatograms extracted from over 1500 injections of bevacizumab performed on a 4.0 mm i.d. × 300 mm long MAbPac SEC-1 column packed with 5 μm macroporous particles applying 100 mM sodium phosphate pH 6.8 in 300 mM NaCl as the mobile phase. Adapted with permission from [58].

**Figure 3.** HIC separation of ADCs having different payload in which the retention time increases with increasing DAR. Adapted with permission from [82].

**Figure 4.** Optimization of an E. coli acetone powders separation in anion-exchange mode by A) a salt gradient with decreasing the slope of the NaCl salt gradient in time, and by B) application of a pH gradient; decreasing the slope of the pH-gradient in the range between pH 3.5-2.4. C) shows the respective salt and pH gradient profiles. For the salt gradients, a 20 mM sodium carbonate buffer at pH 9.7 was used as mobile-phase A and 20 mM sodium carbonate buffer at pH 9.7 containing 1 mM NaCl was used as mobile-phase B. For the pH gradients, a proprietary pISep buffer (mixture of polyionic organic buffering molecules) at pH 2.4 was used as buffer A, and

buffer B consisted of pISep buffer at pH 10.9. The column volume (CV) was approximatively 2 mL. The applied gradient slopes are: $a_1$: 13.6 mM NaCl/CV, $a_2$: 10.9 mM NaCl/CV, $a_3$: 8.0 mM NaCl/CV, $a_4$: 5.0 mM NaCl/CV, $a_5$: 4.3 mM NaCl/CV for the salt-gradient profiles, and $b_{1and}$ $b_2$: 0.1 pH units/CV, $b_3$: 0.1 pH units/CV from pH 9.7-3.5 and 0.05 pH units/CV from pH 3.5-2.4, $b_4$: 0.1 pH units/CV from pH 9.7-3.5 and 0.025 pH units/CV from pH 3.5-2.4 for the pH-gradient profiles. Adapted with permission from [107].

**Figure 5.** Schematic overview of the different state encountered in protein MS analysis.

**Figure 6.** ESI mass spectra of anti-thrombin III (A) acquired under native MS conditions using 20 mM ammonium acetate, (B) using 20 mM ammonium acetate/methanol/formic acid 49:50:5 (v/v/v)%, and (C) denaturing conditions using 20 mM ammonium acetate/methanol/formic acid 45:50:5 (v/v/v)%. Adapted with permission from [147].

**Figure 7.** (A) Native MS spectrum of a deglycosylated mAb (IgG1) yielding a narrow charge envelope situated in the high m/z region and corresponding deconvoluted mass spectrum shown in the inset to determine the intact mass. (B) Subsection of a native MS spectrum from a glycosylated mAb displaying increased mass heterogeneity and corresponding deconvoluted mass spectrum in the inset revealing the presence of different glycoforms. Adapted with permission from [146].

**Figure 8.** Native MS spectra of IgGs (CNTO5825 and NIST) obtained on a Q-TOF-2 instrument (Waters) after direct infusion (A and B) and after SEC analysis, indicating partial unfolding of the antibodies (without breaking disulfide bonds). LC conditions: Flow rate = 0.1 mL/min; mobile phase = 100 mM ammonium acetate, pH 6.8; using a 4.6 mm i.d. × 100 mm BEH SEC column packed with 1.7 μm particles (200Å pores).

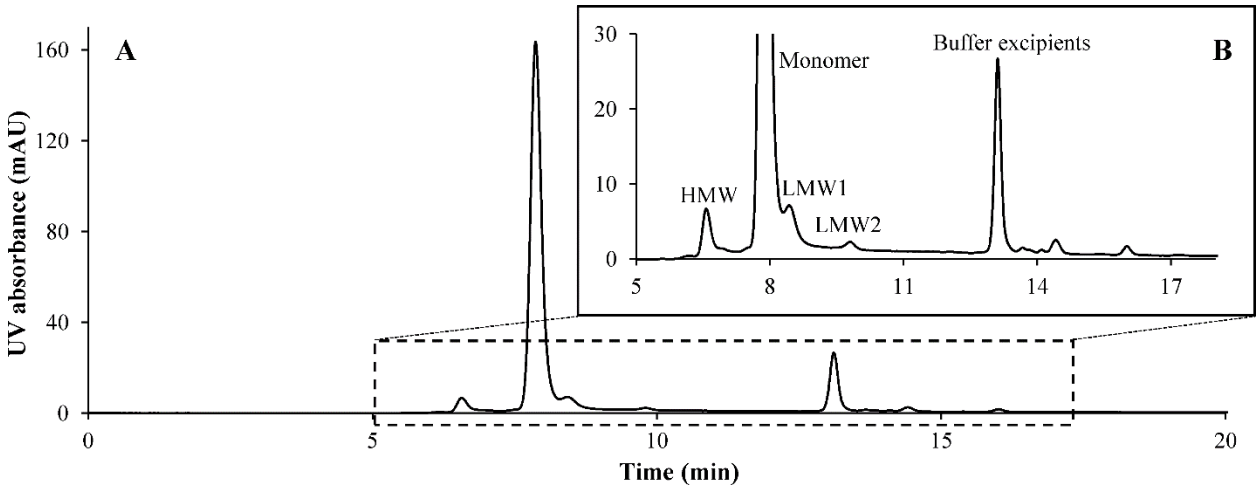

A
B
UV absorbance (mAU)
Time (min)
Monomer
Buffer excipients
HMW
LMW1
LMW2

Figure 1

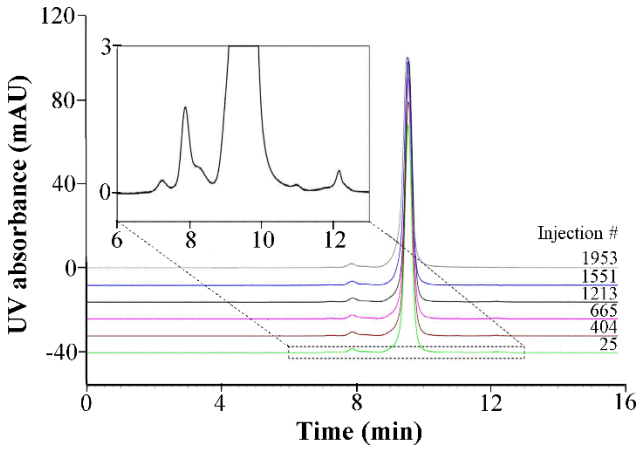

120
80
40
0
-40
UV absorbance (mAU)
3
0
6
8
10
12
Injection #
1953
1551
1213
665
404
25
0
4
8
12
16
Time (min)

Figure 2

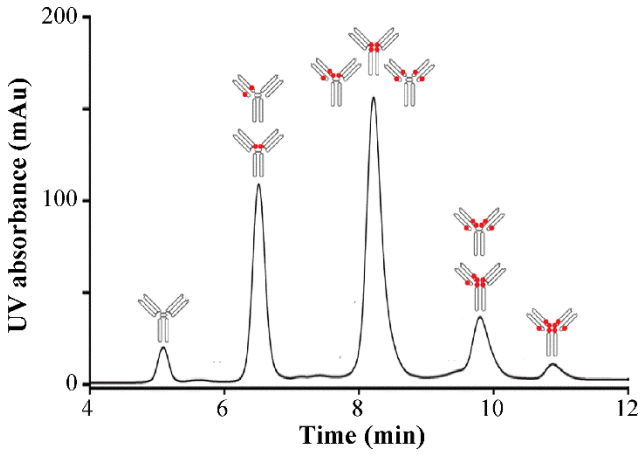
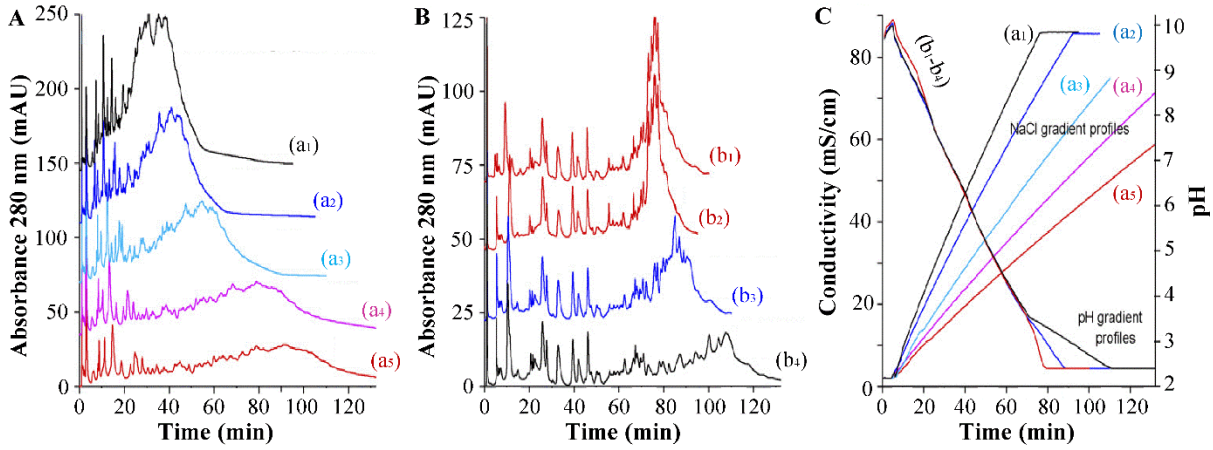


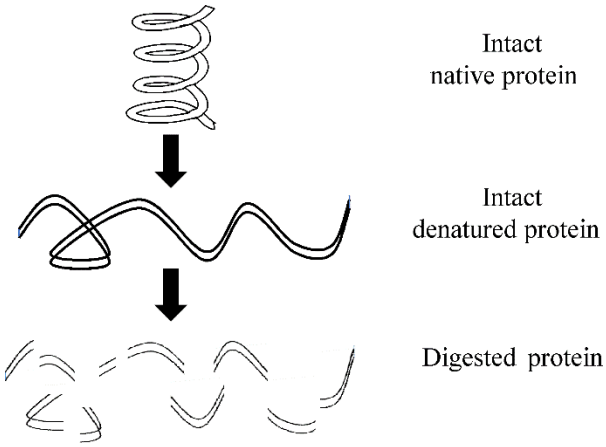

Figure 3




59

60 Figure 4

61




62

63 Figure 5

64

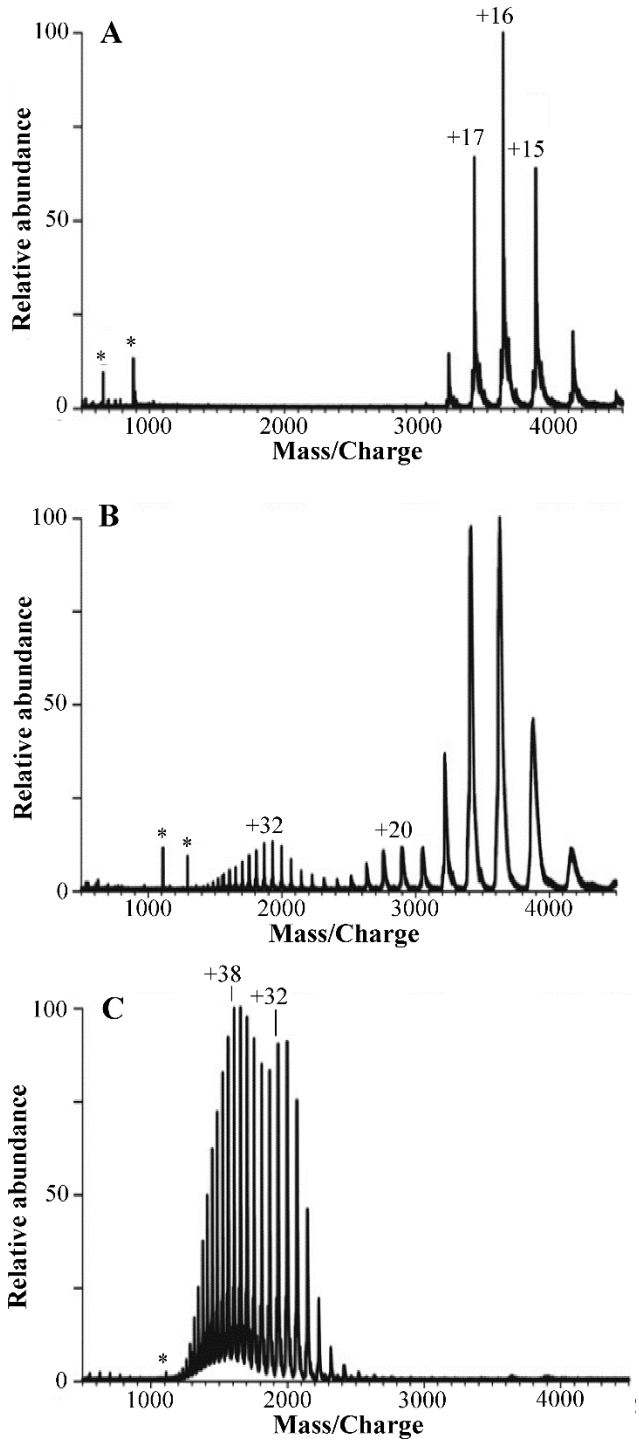
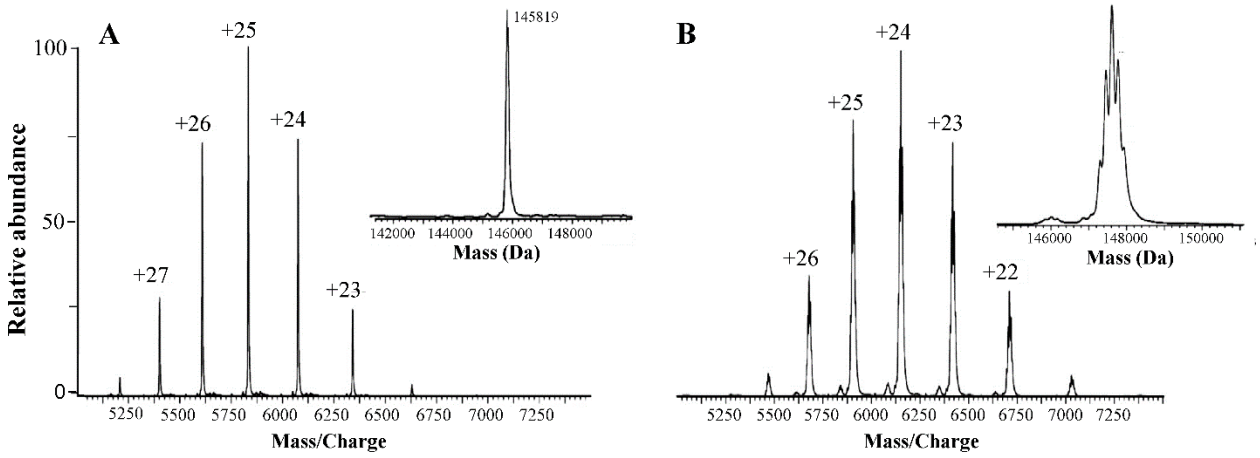

A
+16
+17
+15
100
50
0
Relative abundance
*
*
1000
2000
3000
4000
Mass/Charge
B
100
50
0
Relative abundance
*
*
+32
+20
1000
2000
3000
4000
Mass/Charge
C
+38
+32
100
50
0
Relative abundance
*
1000
2000
3000
4000
Mass/Charge

Figure 6




67

68 Figure 7

69

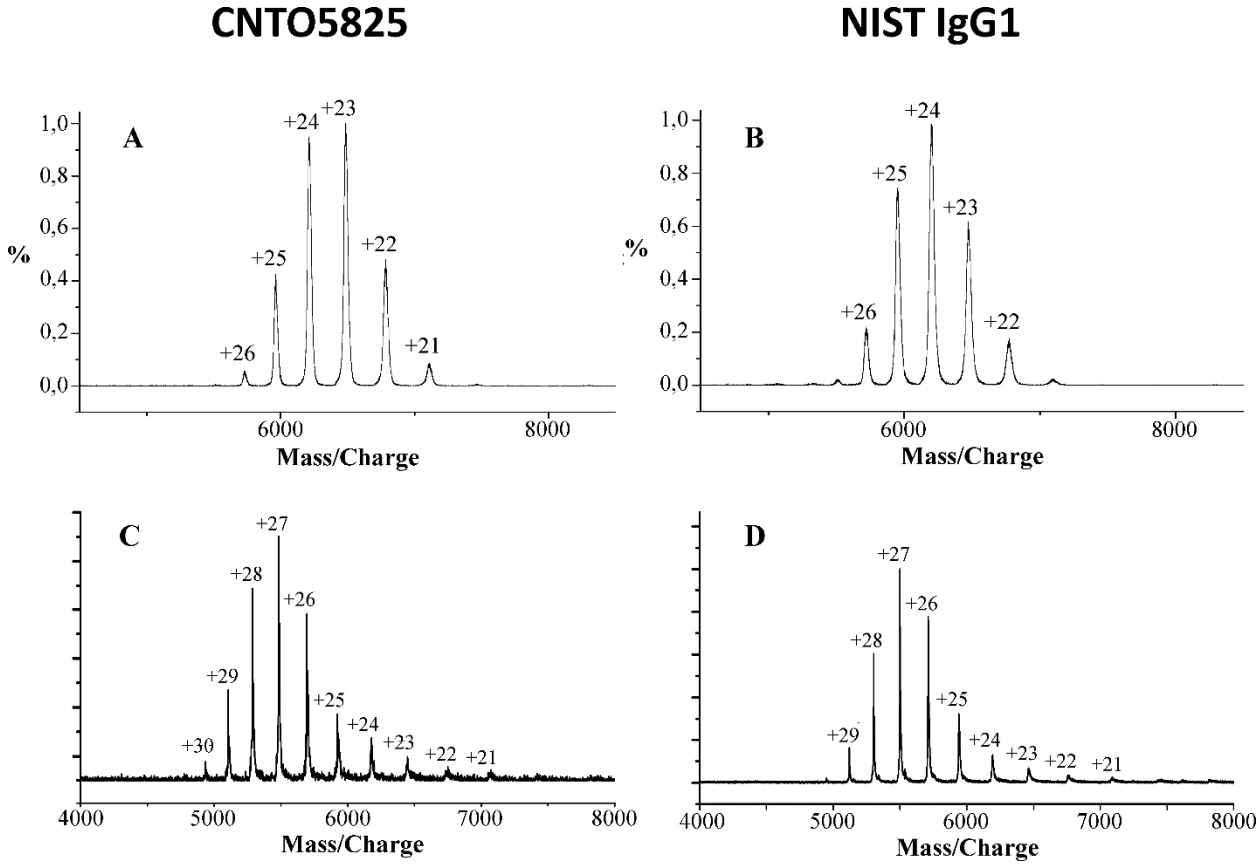

CNTO5825
NIST IgG1
A
B
C
D
%
Mass/Charge
+21
+22
+23
+24
+25
+26
+27
+28
+29
+30

Figure 8