Unraveling proteoform complexity by native liquid chromatography-mass spectrometry

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Studying protein structure and function by native separation - mass spectrometry

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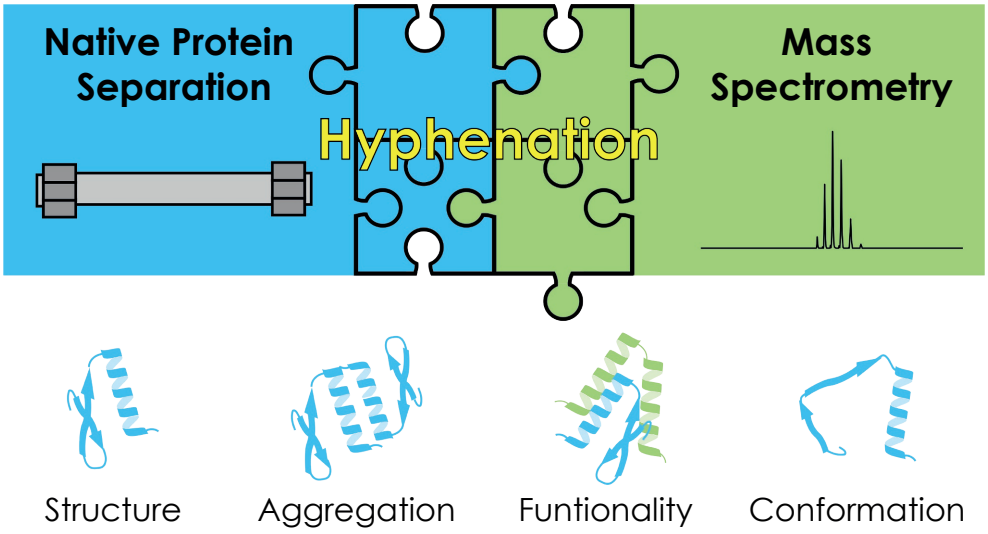
Alterations in protein structure may have profound effects on biological function. Analytical techniques that permit characterization of proteins while maintaining their conformational and functional state are crucial for studying changes in the higher order structure of proteins and for establishing structure-function relationships. Coupling of native protein separations with mass spectrometry is emerging rapidly as a powerful approach to study these aspects in a reliable, fast and straightforward way. This review presents the available native separation modes for proteins, covers practical considerations on the hyphenation of these separations with mass spectrometry and highlights the involvement of affinity-based separations to simultaneously obtain structural and functional information of proteins. The impact of these approaches is emphasized by selected applications addressing biomedical and biopharmaceutical research questions.



2.1 Introduction

Proteins are bioactive molecules that perform key roles in virtually all processes of life. Each biological function is strongly associated with the protein's structure and regulation is often qualified by a structural change. Proteins are heterogeneous species comprised of genetic variants and many post-translational modifications [31]. The various molecular forms of an expressed protein are defined as proteoforms – they are independent of the source of structural variation [28]. The proteoforms can differ in their chemical composition and also their higher order (or spatial) structure, which contributes to functional variability. Most of these changes in protein structure are dynamic and are often part of protein regulation and homeostasis. Accordingly, abnormal proteoforms have been associated with various diseases [58]. For instance, dysregulations in protein phosphorylation have been broadly observed in neurological diseases, type 2 diabetes and cancer [58-60]. Another example is glycosylation, which provides protein stabilization and regulates interaction with cell receptors [61]. Changes in glycosylation have been linked to numerous diseases, including inflammation and cancer [62]. Changes in the shape and conformation of proteins may also cause their destabilization, aggregation and/or tissue deposition, which are associated with 'conformational diseases', such as Alzheimer's disease and Parkinson's disease [63, 64]. Aside from endogenous proteins, recombinant proteins have a pivotal role in our modern lifestyle, with applications in industrial enzymes for bioethanol and food production to therapeutic proteins [16, 65]. Their usefulness and safety strongly rely on the exact chemical constitution and conformation of the protein. For instance, aggregate formation of protein therapeutics is a critical issue, as it can cause reduced efficacy and changes in bioavailability, which may induce immune responses [57]. In addition, protein charge variants may show altered receptor binding, affecting bioactivity and clearance [55]. Clearly, structural and functional characterization of proteoforms is essential and has become indispensable. Yet, it remains one of the main analytical challenges in modern biotechnology and bioanalytical laboratories.

Separation techniques hyphenated with mass spectrometry (MS) are undoubtedly the primary tools for the structural characterization of proteins in complex mixtures. MS-based bottom-up and/or top-down proteomic approaches are extensively used for identification of proteoforms [66, 67]. Whereas these proteomic workflows provide detailed compositional information, they often also induce a loss of the native conformational and functional state of the analyzed proteins, leaving questions open as to their original higher order structure (HOS) (relating to the presence of conformers,

aggregates or complexes) and activity. Native analytical techniques, such as native mass spectrometry (nMS) or native separation techniques (e.g., native liquid chromatography; LC and native capillary zone electrophoresis; CZE), can characterize proteins while leaving their structure, conformation and/or functionality intact. These techniques can be applied under analytical conditions (for example, solvents, buffers, electrolytes and temperature) that do not alter the functional state of the protein. Native LC has been employed for many years for protein purification and for monitoring protein aggregation, non-covalent protein conjugates and protein complexes [68, 69]. However, the compositional and proteoform information derived from stand-alone native separations is limited and, usually, additional methods are needed to identify and characterize individual proteoforms. nMS can provide information on, for example, protein identity, HOS, metal cofactors, binding sites, stoichiometry and/or the exposed surface of proteins or complexes [70]. Unfortunately, signal suppression and overlapping MS signals are often encountered for protein mixtures and heterogenic proteins when analyzed directly by nMS [71]. In such instances, the hyphenation of nMS with native separation is required. Furthermore, as proteins in their native state are better representatives of a biological system, there is a growing interest in the top-down proteomics field to study proteins under more natural conditions. Native top-down proteomics has enormous potential and aims to provide a full representation of proteins and protein complexes in a cell [70]. Yet, due to the complexity of proteomes, attaining such an objective inevitably requires high-resolution native separations prior to nMS detection [72].

Thanks to recent advances in MS instrumentation, separation technology and interfacing options, the hyphenation of native separations with MS is now a reality, and this emerging field is expanding rapidly. MS of proteoforms separated in their native state has been achieved using conventional (i.e., denaturing) and native approaches (**Fig. 1**). Both denaturing and native approaches provide compositional and proteoform-specific information, with the latter permitting investigation of HOS and interactions. Next to unraveling MS signals and facilitating data interpretation, hyphenation of native separations with MS provides unique information to solve questions that cannot be answered by standalone native techniques. For instance, accurate information on protein aggregates is one of the most challenging aspects in nMS, as aggregation can potentially occur during the ionization process due to electrostatic interactions [73]. Similarly, the presence of complexes stabilized by hydrophobic interactions can be overseen with nMS, as they tend to dissociate in the gas phase [74]. The use of native separation techniques prior to nMS discriminates between HOSs present in

solution and electrospray artifacts and can address protein interactions of gas-phase unstable complexes. Moreover, the identification of metastable conformations of proteins with subtle structural differences – which is not attainable by comparing their mass spectra – can be resolved by separation techniques [75].

In this review, we discuss the recent advances on the hyphenation of native protein separations with MS for the assessment of protein composition and (spatial) structure. The state of the art is provided, giving a comprehensive overview of the native methods reported, also covering practical considerations to their application. Furthermore, we highlight the combination of affinity-based separations and MS to simultaneously obtain compositional, structural and functional protein information.

2.2 Practical considerations

Although the online coupling of native separation techniques with MS provides clear benefits, it also brings practical challenges. The analytical conditions must safeguard that protein structure is preserved while adequate separation of protein species is attained. Protein separation techniques are combined with MS using electrospray ionization (ESI), permitting direct ionization of (macro)molecules from the separation effluent and delivering a distribution of multiply charged gas ions into the mass spectrometer [76]. In ESI, charged droplets are generated by applying a high voltage at an electro-spray needle containing the flowing solution, followed by solvent evaporation (or desolvation), charge transfer and gas-phase ion formation [77-79]. ESI is a soft ionization technique ensuring that protein structure and non-covalent interactions are maintained when native conditions are applied. Solvent systems commonly used for native protein separations, however, are often not (fully) compatible with ESI-MS. Generally, significant concentrations of salts are required to achieve good protein resolution while preserving their HOS [80, 81]. Phosphate buffers and sodium chloride gradients that are used in many native LC separations are typically employed in high concentrations [82, 83]. The presence of these non-volatile salts during ESI may cause (1) severe protein ionization suppression, (2) unwanted adduct formation and (3) contaminating salt deposits, which can all seriously compromise the performance of the MS instrument [78, 84]. Protein ionization suppression, in particular, reduces ionization efficiency, thereby, drastically decreasing detection sensitivity [85]. Moreover, adduct formation of salt and protein ions causes the distribution of the MS signal over multiple species, which reduces sensitivity, increases spectral complexity, as well as hindering the ability to

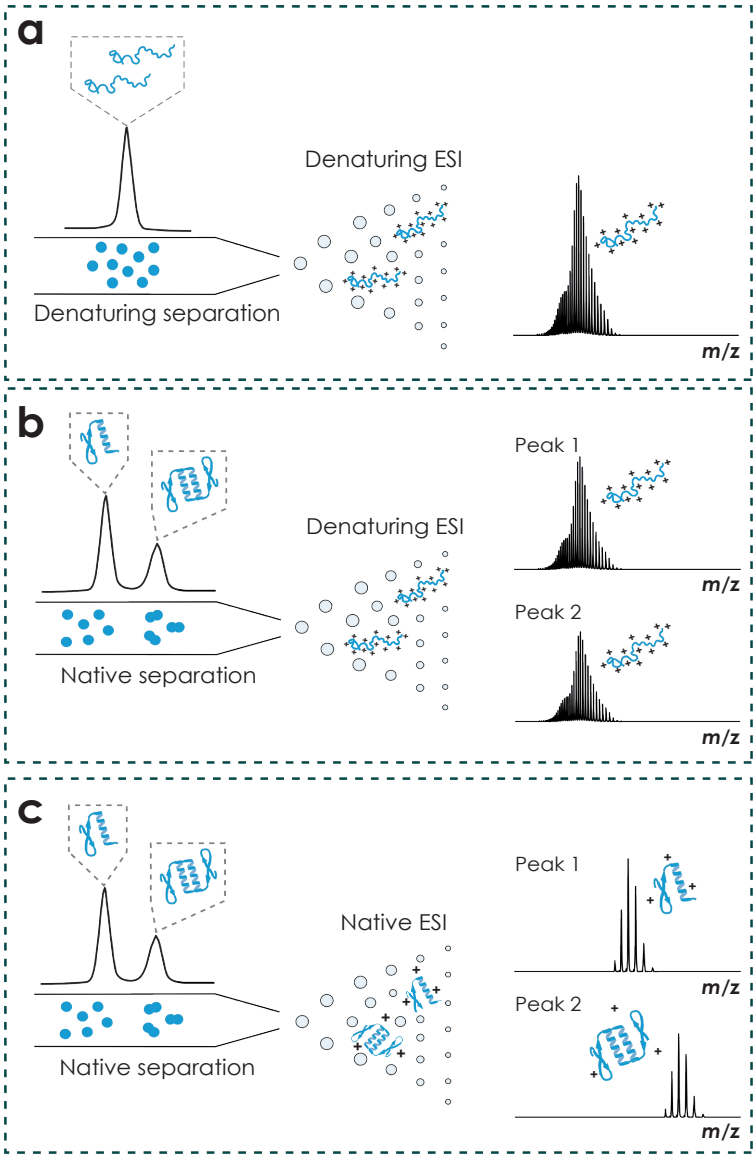


Figure 1 | Representation of the implications of denaturing and native protein analysis exemplified by a protein and its non-covalent dimer. (**a**) Denaturing analysis as a combination of both denaturing separation and denaturing ESI-MS. Denaturing separation induces unfolding of the protein and results in loss of the HOS. In this particular example, a sample composed of a monomer (single dot) and a homodimer (double dots) results in a single peak due to dissociation of the homodimer. (**b**) Native separation combined with denaturing ESI-MS. Here, the denaturing of the proteins is induced after the separation during the ionization process. While the HOS of the homodimer is preserved during separation, the denaturation during ionization leads to unfolding of the proteins and, therefore, mass spectra exhibiting many charge states. Although the protein and the homodimer are separated, only their monomeric forms are detected in MS. (**c**) Native analysis using both native separation and native MS detection. This scheme exemplifies a complete native analysis typically resulting in native mass spectra exhibiting fewer charge states to permit the detection of non-covalent assemblies. Created with BioRender.com.

distinguish and identify proteoforms with only minor differences in mass [86, 87]. To overcome these problems, hyphenated native separation MS workflows often require adaptations compared with conventional native protein separations, as described in the following paragraphs.

In hyphenated separation MS workflows, volatile electrolytes, such as ammonium acetate, formate or bicarbonate buffers, are primarily used. These salts still allow protein ionization when used at concentrations between 20 and 200 mM, and, in some cases, even up to 500 mM [67, 88, 89]. The protonating agent present in these buffers (NH_4^+) is removed during the ionization process via NH_3 evaporation, largely circumventing adduct formation [78, 90, 91]. By using ammonium acetate, protein conformation is normally preserved during both the separation and the ESI process, whereas formate and bicarbonate may cause protein denaturation [92]. With bicarbonate buffers, CO_2 bubble formation can occur during the ESI process, potentially inducing protein unfolding and protein complex disruption [93]. The pH of the separation medium may affect protein activity and HOS, and, therefore, is an important parameter to consider when native analysis is pursued. In general, the use of a separation pH equal or close to the isoelectric point (pI) of proteins should be avoided as protein solubility might be low, leading to unwanted aggregation or adsorption [67]. As most proteins exhibit their optimum activity at physiological pH, the use of ammonium acetate as salt for separation of proteins prior to MS detection is often recommended. An aqueous solution of ammonium acetate presents a neutral pH (6.5–7), although it should be noted that such a solution shows maximum buffer capacities around the pK_a of ammonium (9.25) and acetic acid (4.75) [67, 81].

From an MS hyphenation perspective, the flow rate at which the separation buffer with analyte proteins is delivered to the ESI source of the MS instrument is an important parameter. One key process during ESI is the spray formation of charged droplets, followed by droplet desolvation and gas ion formation [78, 79]. The initial size of spray droplets increases with flow rate, in which small droplets favor the desolvation of proteins. A conventional ESI spray needle with a diameter of 0.5 mm typically provides a stable spray at flow rates of several microliters per minute or more [94]. Modern ESI sources show optimal ionization performances at flow rates between 0.001 and 0.1 ml/min; however, they can accommodate up to 1.0 ml/min of flow [95]. Typical flow rates employed in conventional protein LC using 2–5 mm inner diameter columns range between 0.1 and 1.0 ml/min. Considering ESI efficiency, flow rates for native LC-MS analysis preferably should not be higher than 0.4 ml/min, with lower flow rates resulting in better sensitivity [96]. For example, the reduction of

the flow rate from 0.25 to 0.10 ml/min can provide a five-fold increase in MS signal intensity [97]. However, reduction of flow rate will be accompanied by a proportional increase of analysis time. Moreover, the lower flow rates often lead to a loss of separation efficiency (that is, higher column plate heights). Therefore, when using lower flow rates, the inner diameter of the LC column should be reduced accordingly. While the usefulness of columns with inner diameters below 2 mm for protein analysis has been demonstrated under denaturing conditions (for example, using reversed-phase LC), native LC of proteins using these columns has been reported to a much lesser extent [98, 99]. Moreover, protein sample loadability decreases with reducing column inner diameter.

In practice, favorably low flows for ESI-MS after protein LC are often achieved by post-column approaches (**Fig. 2**), such as flow splitting, which allows the use of high flow rates for separation, while transferring only a fraction of the effluent to the MS instrument [100, 101] (**Fig. 2b**). This is particularly useful when high sensitivity is needed, for instance, when detection of low abundant species, such as minor variants or endogenous proteins, is required. Substantial flow splitting, in principle, would also permit use of a nano-ESI source, which provides very small spray droplets and enhanced protein desolvation, resulting in ultimate MS sensitivity [102]. Nano-ESI is possible for flow rates from a few nl/min up to 1 µl/min. Next to favorable ionization efficiency, soft ionization and low background signals (this is, increased signal-to-noise ratios) [78, 87, 90, 95], nano-ESI also exhibits a greater tolerance towards ionization suppression by non-volatile salts [77, 87, 94, 103]. In the field of native LC-MS, the use of nano-ESI is still quite uncommon but reported applications of analytical protein LC separations coupled to nano-ESI by a post-column splitter have demonstrated its feasibility [73, 89, 102]. As CZE employs very low flow rates (typically below 1 µl/min), direct coupling of CZE with nano-ESI (that uses sheathless CZE-MS or an electrokinetically driven sheath liquid interface) is possible [80, 104-107].

In order to achieve adequate hyphenation between native separation and MS, several approaches can be followed, depending on the separation buffer used (**Fig. 2**). In general, native separations using low-ionic-strength volatile buffers and low to moderate flow rates can be directly coupled with MS (**Fig. 2a**). Low concentrations of volatile salt might not provide optimal chromatographic separation of target proteins. For instance, low-ionic-strength mobile phases in some chromatographic systems might not sufficiently suppress undesirable electrostatic interactions of proteins with the stationary phase [89, 92, 108]. When significant concentrations of volatile salts are required for separation, direct coupling is still possible, but often at a loss of

sensitivity. In such cases, compatibility and sensitivity can be enhanced by post-column strategies, such as reduction of the flow to the MS instrument by flow splitting, enrichment of the protein desolvation gas with vapor of an organic solvent (for example, isopropanol or acetonitrile) or addition of a make-up solvent to the effluent. The use of a post-column flow splitter is relatively straightforward and results in more efficient protein ionization to compensate for the adverse effects of the high ionic strength (**Fig. 2b**). This strategy is mainly beneficial for native LC-MS methodologies using moderate to high flow rates. Protein ionization conditions could also be improved by saturation of the nitrogen gas that flows around the ESI emitter with organic vapor ('dopant gas') using a dedicated gas supply (**Fig. 2c**). This may be employed to alleviate protein ionization suppression and undesired adduct formation [89, 109]. The use of a post-column make-up flow may decrease the salt concentration prior to protein ionization with either a volatile buffer solution (allowing nMS) or an organic solvent (inducing denaturing MS) [88]. Both approaches aim to circumvent compatibility issues by enhancing protein ionization (**Fig. 2d**).

The use of non-volatile buffer salts for native separations may be mandatory in order to attain the required protein resolution. MS-compatible buffers can also alter the protein activity or the stability of protein complexes. For example, *Escherichia coli* ribosomal proteins and protein complexes require the presence of Mg^{2+} ions in solution to maintain their intact structure [110]. When the concentration of non-volatile salts in the eluent is sufficiently modest, a post-column diluting make-up flow may alleviate the problems associated with too high concentrations of salt, allowing MS detection. When concentrations of non-volatile salts are too high or infusion of non-volatiles into the mass spectrometer is not desirable, a desalting or buffer exchange step is needed prior to the MS detection. This can be achieved in an online fashion using a 2D LC strategy, in which a volatile buffer is applied in the second dimension of the native LC [100, 111, 112] (**Fig. 2e**). Moreover, online membrane ion suppressors may also be useful for this purpose. However, the utility of this approach is only to remove trifluoroacetic acid from denaturing LC eluent prior to ionization [113, 114] (**Fig. 2f**).

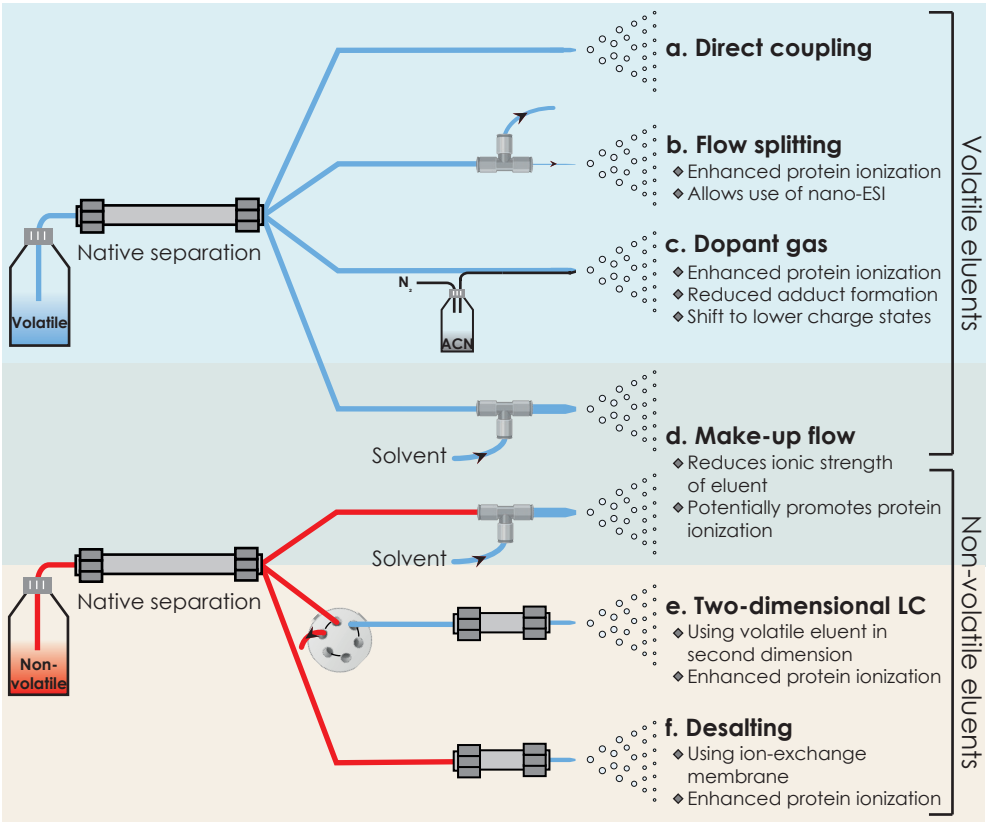


Figure 2 | Hyphenation of native protein separations with MS when using volatile or non-volatile eluents. The most straightforward way of hyphenating native separation techniques with MS is direct coupling, which is recommended when using low-ionic-strength volatile separation buffers [115] (**a**). When high-ionic-strength volatile buffers are required for the separation, post-column manipulation can be considered to facilitate sensitive protein mass spectrometry detection. Conceivable post-column approaches: reduction of flow to ESI source using flow splitting [73] (**b**); use of dopant gas to enhance protein ionization conditions [89] (**c**); introduction of make-up solvent to dilute the buffer concentration and/or improve protein ionization [88] (**d**). The latter approach may also be used when low concentrations of non-volatile buffers/salts are employed for protein separation. Coupling of native protein separations using high concentrations of non-volatile buffers/salts can be accomplished by involving a second-dimension separation (e.g., using size-exclusion chromatography) [111] (**e**) or an ion-exchange membrane step for desalting (**f**). Thicker lines represent higher flow rates. The T-junctions were created with BioRender.com.

2.2.1 Mass spectrometry for native protein analysis

MS analysis of native proteins and protein complexes should be performed with suitable MS instrumentation. Whereas the charge-state distributions are broad in the mass spectra of denatured proteins, they are generally narrower for folded protein structures at higher mass-to-charge ratios (*m/z)* (that is, lower charge states) [116, 117] (**Fig. 1**). Therefore, mass analyzers with adapted

acquisition ranges are required to detect high *m*/z values [110]. Other critical factors in nMS of large proteins and protein complexes are efficient ion desolvation to remove volatile salts from electrospray droplets, transmission of high *m*/z ions to the analyzer and high mass resolution to resolve small mass difference (that is, proteoform resolution) [118, 119]. Examples of suitable mass analyzers are time-of-flight (TOF), Fourier-transform ion cyclotron resonance (FTICR) and Orbitrap devices with extended mass ranges [110, 120-123]. TOF instruments have been extensively employed due to their rather stable resolving power with increasing *m*/z, whereas for FTICR and Orbitraps, their resolving power decreases with increasing *m*/z [118]. Of note, the use of a mass analyzer with very high resolving power will often not pay off in nMS, as the width of the native protein signals as determined by isotopic distributions and residual salt and solvent adducts will limit effective resolution. FTICR-MS instruments provide excellent resolution of protein ions. However, their accessibility is limited due to the elevated instrument cost. Recently, a shift towards Orbitrap instruments has occurred, as they provide high sensitivity and resolution, even at high *m*/z [124]. Current Orbitrap MS systems provide more efficient desolvation compared with FTICR and TOF instruments due to the possibility to store the ions in a high-energy collision dissociation (HCD) cell at a higher gas pressure [125]. A detailed overview and guidelines for nMS of proteins or protein complex analysis can be found elsewhere [119, 126].

Fragmentation of ions of native proteins and protein complexes in the mass spectrometer can provide information on protein identity (native top-down) and sub-units (complex-up and complex-down) [127]. Fragmentation can be achieved using tandem MS (MS/MS), where particular *m*/z values of precursor ions are selected, followed by ion activation and fragmentation. Collisional activation approaches (for instance, collision-induced dissociation and HCD) are available on most mass spectrometers and enable the dissociation of protein complexes (complex-up). However, backbone fragmentation of proteins or subunits is often inefficient, requiring alternative fragmentation approaches [72]. Electron-transfer dissociation and ultraviolet (UV) photodissociation provide higher sequence coverages for monomeric proteins (native top-down) [119]. Recently, combinations of fragmentation approaches have shown complementarity [110]. An example of such a hybrid technique is the use of HCD for protein complex dissociation into subunits, followed by isolation and UV photodissociation fragmentation of the resulting monomeric species (complex-down) [110, 128, 129]. More information on ion activation technologies and fragmentation possibilities for native top-down proteomics can be found in dedicated reviews [130, 131].

Recently, mass spectrometers equipped with an ion mobility (IM) cell between the ionization source and the analyzer have been released. IM is a gas-phase separation technique that provides information on protein conformation, topology and geometry [132, 133]. In the IM cell, the gas ions are separated based on their charge, size and shape due to their different mobility through the neutral drift gas when an electric field is applied [77]. Gas-phase separation can be useful to reduce sample complexity and provides information on molecules with identical *m/z* values, yet different shape and size [123]. Additionally, strategies combining gas-phase activation and IM-nMS, such as collision-induced unfolding, have gained attention to assess gas-phase stability of proteins [134, 135]. Here, the collision voltages are increased in the trap cell prior to the IM separation, leading to activation and conformational changes of the proteins, including unfolding. For further reading on this topic, see the reviews [123, 136].

2.3 Native separations hyphenated with mass spectrometry

Proteoforms – the myriad of protein products arising from a single gene – may exhibit quite diverse physico-chemical characteristics. There is no single analytical technique able to universally address a complete set of proteoforms and their properties. Protein separation techniques exhibiting different selectivities are available and can be selected to distinguish proteoforms based on, for example, their (relative) size, charge, hydrophobicity, hydrophilicity, conformation or (composition of) post-translational modifications. Protein separation modes – in particular, size exclusion chromatography (SEC; protein size), ion-exchange chromatography (IEX; protein charge), hydrophobic interaction chromatography (HIC; protein hydrophobicity) and CZE (protein charge/shape) – can permit separation and characterization of proteins and protein complexes under native and MS-compatible conditions. Each of these native separation techniques hyphenated with MS can obtain different types of information and also provide a typical proteoform separation profile and corresponding mass spectra (**Fig. 3**). A direct comparison between various native LC and CZE separations is provided in **Table 1**.

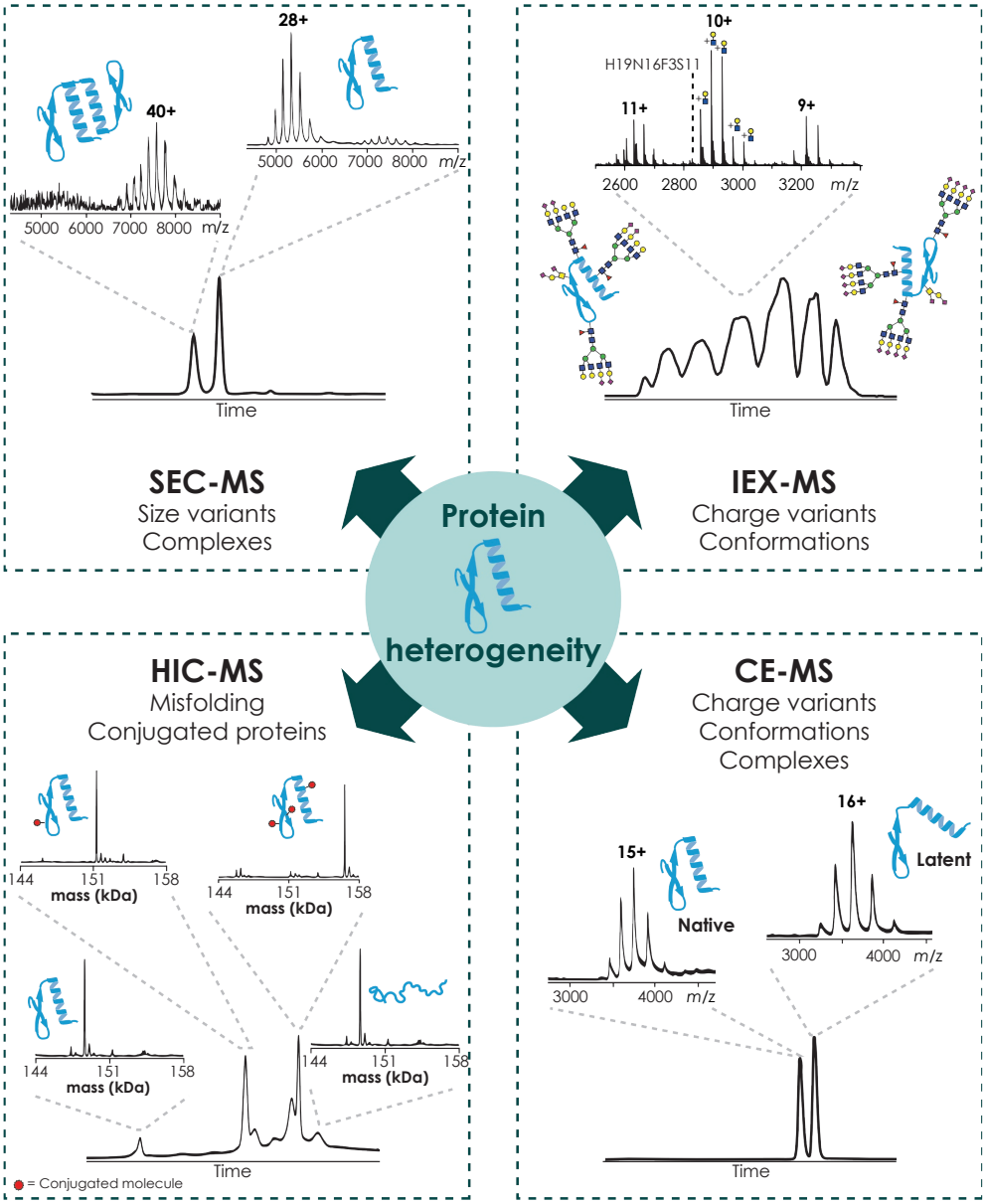


Figure 3 | Scope of native protein separation-MS techniques for the assessment of proteoform heterogeneity. Each hyphenated technique yields specific information on particular protein characteristics, as illustrated by a typical proteoform separation with corresponding mass spectra. SEC can provide separation of protein size variants, such as aggregates, fragments or complexes. IEX can provide resolution of protein charge variants, such as glycoforms differing in number of sialic acids. HIC separates proteoforms exhibiting differences in hydrophobicity as result of, for example, misfolding or conjugation with drug molecules. CZE can provide separation of protein charge variants, complexes and conformers, such as active and latent forms of a protein. SEC-MS adapted with permission from [111]. IEX-MS adapted from [101]. HIC-MS adapted with permission from [100]. CZE-MS adapted with permission from [75].

Table 1 | Comparison of the characteristics of native separation techniques coupled to MS for proteoform analysis.

Analytical aspects	SEC-MS	IEX-MS	HIC-MS	CZE-MS
Selectivity	Size variants	Charge and/or conformational variants	Hydrophobic variants	Charge, conformational and/or size variants
Commonly targeted proteoforms	Aggregates, complexes and fragments	PTMs altering charge (*e.g.*, phosphorylation, deamidation) and conformers	PTMs changing hydrophobicity (*e.g.*, oxidation), misfolding and conjugated proteins	PTMs (*e.g.*, phosphorylation, deamidation), complexes and conformers
Sensitivity	+/-	+/-	-	+
Robustness	+	+	+/-	-
Suitability for minute samples	-	-	-	+
Suitability for affinity analysis	+/-	-	-	+

2.3.1 Size exclusion chromatography

SEC separates proteins based on their differences in hydrodynamic volume, which is strongly related to their molecular size. The combination of SEC and MS is particularly useful for the detection and characterization of protein aggregates (for example, dimers and trimers) or protein fragments of lower molecular weight [137] (**Fig. 3**). For instance, size variants of therapeutic proteins can be distinguished by SEC-MS, which is essential, as they may be highly immunogenic [73]. The native running conditions of SEC are key, as most aggregates are linked by non-covalent interactions and, therefore, would disassemble under denaturing conditions. Moreover, SEC-MS separation of (non-covalent) oligomeric species from the parent protein reveals whether they were present in the analyzed solution or were formed during the ESI process [73, 138].

The separation mechanism of SEC differs from regular chromatographic techniques, as retention is governed by size-dependent protein exclusion from or diffusion into the pores of stationary phase particles (entropy-driven) instead of interactions with the column material (enthalpy-driven) [139]. The pore-size distribution of the particles is the principal parameter to determine

protein-size resolution [97]. Protein molecules that are sufficiently small enough to enter most of the pores have the strongest chromatographic retention, whereas proteins that are too large to diffuse into the pores elute the fastest. Currently, protein SEC columns are available with average pore sizes in the 125-900 Å range, in which pore diameters between 200 and 300 Å provide separation for proteins with a molecular weight between 10 and 500 kDa [140, 141]. As elution, in principle, does not depend on molecular interactions, SEC typically employs isocratic conditions, with the mobile phase acting merely as carrier solvent. Traditional protein SEC mobile phases consist of aqueous phosphate buffers (5-50 mM) with sodium or potassium chloride (up to 250 mM) [111, 142]. The substitution of these non-volatile electrolytes for volatile MS-compatible buffers and salts is not trivial, as the nature, ionic strength and pH of the mobile phase can greatly impact protein retention [87, 89]. For instance, the use of ammonium acetate in mobile phases may result in longer protein retention times (causing an underestimation of high-molecular-weight species), peak broadening, increased adsorption and/or reduced recovery [111, 142]. These unwanted effects are mainly caused by so-called secondary interactions with the stationary phase, which can be polar, electrostatic or hydrophobic in nature [143]. The nature of the column material can alleviate the presence of secondary interactions. For instance, derivatized silica-based column materials are preferred for protein analysis, as they shield residual, negatively charged silanol groups, which reduce interactions with positively charged proteins (for example, ethylene-bridged hybrid inorganic-organic materials) [89, 97, 144, 145]. Additionally, an increase in ionic strength of the mobile phase (> 100 mM) can help to reduce ionic interactions of proteins and their aggregates to residual charges on the column material surface [92]. In particular cases (for example, for separation of proteins with similar size) [145], secondary interactions may be used to enhance resolution. This mixed-mode SEC-MS is promising; however, its application has been limited.

For protein sample desalting purposes [146], short SEC columns (< 50 mm) with relatively small pore diameters (< 200 Å) are used, allowing fast separation of protein molecules from electrolyte ions, while limiting sample dilution [97, 133, 134, 147, 148]. Protein separations require much longer columns (150-300 mm) to achieve resolution of size variants. As the maximum protein elution volume cannot exceed one column volume, the use of relatively wide columns (inner diameter between 6 and 8 mm) of sufficient length is required to attain good separation. However, this leads to long retention times and significant peak broadening [97]. To alleviate this drawback, ultrahigh-performance SEC has been introduced, which employs columns with an inner diameter of 4.6 mm packed with sub-3 µm particles to obtain shorter analysis times without

compromising protein resolution [140, 141]. Of note, the accompanying high flow rates may cause frictional heat and shear stress, potentially resulting in protein degradation or loss of HOS, such as aggregates [137, 147, 149, 150].

Complementary information on conformation, stability or affinity of protein species separated by size may be obtained by incorporating IM in native SEC-MS approaches [97, 111, 134, 151]. SEC-IM-nMS, in principle, can yield solution and gas-phase structural data in a single experiment. In addition, SEC collision-induced unfolding has been reported as a suitable approach to study the unfolding behavior of proteins [134, 150]. This relatively new approach reduces the time of sample preparation (as it is an online instead of a manual buffer exchange) and of data collection (from 3 h to 15 min) compared with direct infusion nano-ESI experiments [134].

2.3.2 Ion-exchange chromatography

IEX is an established technique for the native separation of protein charge variants, which result from, for example, deamidation, pyroglutamation, carbamylation and aspartic acid isomerization [82]. These modifications alter the overall charge and local charge distribution of proteins, which may lead to altered receptor binding or half-life in serum [152, 153]. In some cases, IEX can also differentiate between native and (partly) denatured proteins, as surface charges may change when residues are exposed to water on unfolding [154] (**Fig. 3**). In IEX, retention is based on electrostatic interactions between the net positively or negatively charged protein analytes (depending on their pI) and immobilized ionic groups (such as strong or weak acidic/basic moieties) on the stationary phase surface, permitting analysis of basic and acidic proteoforms. Strong cation exchangers (CEX) and anion exchangers (AEX) are negatively or positively charged, respectively, over a broad pH range, making them generally applicable. The effective charge of weak ion exchangers strongly depends on the adjusted pH, which limits their applicability but also presents the option to achieve specific selectivities [82]. Highly cross-linked poly(styrene-divinylbenzene) is a common stationary phase material for IEX, as it exhibits high stability over a wide pH range and good pressure resistance [101]. Stationary phase particle sizes of 3-10 µm are often used (sub-2 µm IEX materials are also available) [82]. Porous particles can be used; however, for protein separations, non-porous particles may be preferred in order to decrease peak broadening caused by the low diffusion coefficients of proteins, which slow down mass transfer between mobile and stationary phases [82]. However, due to the reduced stationary phase surface, non-porous materials have limited sample capacity and provide less retention.

Straightforward coupling of IEX and MS can be achieved using aqueous mobile phases comprising volatile salts (for example, 35 mM ammonium formate, pH 7.4) and applying isocratic elution [154]. In IEX, however, salt gradients – in which the salt concentration of the mobile phase is increased over time – are generally preferred for elution to prevent very long analysis times and to achieve high separation efficiencies for complex protein samples. Conventional IEX eluents containing non-volatile phosphate buffers and sodium chloride gradients (up to 1 M) are typically replaced by ammonium acetate gradients (50 to maximal 600 mM) in order to allow for gradient IEX-MS [96, 155, 156]. However, even high concentrations of volatile salts may compromise MS detection and sensitivity, potentially hindering the characterization of low-abundant proteoforms. Therefore, in IEX-MS, the use of pH gradients based on low-ionic-strength mobile phases (20-50 mM) has gained interest. The elution of proteins from IEX phases is promoted when the pH of the mobile phase approaches the pI of the proteins. CEX-MS employing volatile mobile phases with gradually increasing pH is used to characterize basic proteins, such as monoclonal antibodies (mAbs) [115, 157-161]. By contrast, pH-gradient AEX-MS of acidic proteins (pI < 6.5) has been rarely reported [162]; however, its potential was shown by separating highly sialylated glycoproteins [101]. IEX-MS using pH gradient elution is robust and reproducible [101, 115, 162]; however, for some proteins, the low-ionic-strength mobile phase may give rise to broad peaks and reduced separation efficiency [108]. In such cases, volatile, salt-mediated pH gradients may improve separation performance [102, 163, 164] for, in particular, mixtures comprising proteins over a wide range of pI values or complex protein species consisting of many different proteoforms, including heavily glycosylated proteins [108]. In this approach, a volatile salt gradient (for example, 50-200 mM ammonium acetate) is superimposed on the pH gradient [165], achieving enhanced separation while largely circumventing loss of protein ionization efficiency by too high ammonium acetate concentrations [163].

2.3.3 Hydrophobic interaction chromatography

HIC is a widely recognized technique for native separation of proteins based on their difference in hydrophobicity. HIC can resolve misfolded and conjugated proteins, but also various post-translational modifications of proteins, which give rise to changes in hydrophobicity [137, 166-168] (**Fig. 3**). This resolution is very useful as, for example, the reliable determination of the degree of conjugation is critical for particular therapeutic proteins [88], and oxidation may impact activity and stability (that is, shelf life) of industrial and pharmaceutical proteins [169]. HIC stationary phases consist of silica-based or

polymer-based particles with moderately hydrophobic ligands (for example, short n-alkyl or phenyl groups) linked to their surface in relatively low density [168]. Protein retention in HIC relies on hydrophobic interactions, which are regulated by varying the concentration of a salt-induced precipitation electrolyte (commonly, ammonium sulfate) in the aqueous mobile phase. Hence, HIC is a native separation technique, in which, as for protein elution in principle, no organic solvent is needed and a concentration-decreasing salt gradient (for example, 1.8-0.02 M ammonium sulfate) [170] can be used, maintaining protein integrity.

Achieving HIC-MS of proteins is not trivial, as commonly used non-volatile eluent salts can have strong adverse effects on the MS performance. Exchanging ammonium sulfate for ammonium acetate often leads to insufficient protein retention, as acetate is a weaker ion to induce precipitation than sulfate [98, 99]. Moreover, very high concentrations of ammonium acetate (up to 3 M) are often required, strongly reducing MS sensitivity. To increase protein retention for ammonium acetate mobile phases, more hydrophobic HIC stationary phases employing ligands of longer alkyl chains have been proposed. Yet, these new HIC materials often require the addition of organic solvent to the eluent for proper protein elution, which is undesired when native separation is desired [99]. For a confined HIC separation of proteoforms with minor differences in hydrophobicity, such as oxidized variants of mAbs, isocratic elution using a medium-ionic-strength MS-compatible mobile phase (for example, 150 mM ammonium acetate) has been demonstrated [171]. Unfortunately, such an approach lacks general applicability. The use of ammonium tartrate in the HIC mobile phase is a promising option. Similar protein separation profiles were obtained when compared with using ammonium sulfate; in addition, ammonium tartrate provides minimal protein signal suppression and adduct formation during MS detection [170]. Another potential HIC-MS strategy involves online post-column dilution of a highly concentrated ammonium acetate mobile phase followed by flow reduction using flow splitting and successive hyphenation with nano-ESI [88] (**Fig. 2**). When diluting the eluent by six times with a flow of water followed by subsequently splitting the flow (about 500 to 1), sensitive and adduct-free MS detection of proteins was achieved without significant chromatographic peak broadening, as demonstrated for the HIC separation of mAbs and antibody-drug conjugates (ADCs). This setup holds great potential as a more general approach for online HIC-MS and could allow the use of non-volatile salts in HIC mobile phases.

The challenges associated with the direct coupling of native HIC with nMS have stimulated the development of solutions based on 2D LC. In these HIC-

SEC-MS approaches, the first HIC dimension employs a conventional eluent comprising a non-volatile salt, while the second SEC dimension provides protein fraction desalting using an MS-compatible mobile phase [100, 112]. The transfer of protein-containing effluent fractions from the first to the second dimension can be accomplished by either heart-cutting (that is, transferring one or a few specific fractions to SEC) or comprehensively (that is, transferring fractions covering the full HIC separation to SEC) using column-switching tools. The implementation of heart-cutting methods is more straightforward, and there are no or only limited time constraints for the second-dimension analysis, potentially permitting higher SEC resolution [112]. Comprehensive HIC-SEC-MS is technically more complex and requires very fast SEC separations (<1 min); however, it allows MS detection of the complete HIC chromatogram, which is beneficial when profiling complex protein samples containing unknown species [100]. The 2D LC approaches allow interference-free protein MS detection; however, they also require protein peak dilution and, therefore, the detection of low-abundant proteins, such as glycosylated proteoforms, can still be challenging [112].

2.3.4 Capillary zone electrophoresis

CZE-MS has proven to be particularly valuable for resolving proteins and proteoforms that exhibit (small) differences in charge. As protein electrophoretic mobility in free solution is proportional to the protein charge-to-size ratio, CZE-MS can also quite uniquely probe differences and changes in protein shape and conformation (**Fig. 3**). This detection can be highly useful, as altered protein folding may cause protein destabilization and aggregation, a phenomenon that has been associated with 'conformational diseases', such as Alzheimer disease or amyloidosis [104]. CZE separations of proteins are obtained in aqueous solutions present in a fused-silica capillary (50-75 µm inner diameter; 20-100 cm length) across which a high voltage (up to 30 kV) is applied. As no stationary phase (and, therefore, no protein interactions) or organic solvent is needed, native separation conditions can be accomplished in a straightforward manner using MS-compatible background electrolytes (BGEs), such as 50-100 mM ammonium acetate or bicarbonate, at (near-)physiological pH [72, 172, 173]. CZE requires only minute amounts of sample, which makes it especially attractive for sample-limited applications, such as clinical protein analysis and native top-down proteomics [80], and also allows repeated analyses of the same sample. Analytical native LC-MS analyses typically require between 50 and 100 µg protein per measurement, whereas CZE injected protein amounts are usually below 1 µg. In order to enhance concentration sensitivity, in-capillary electrophoretic

sample preconcentration and stacking strategies may be applied, allowing injection of up to 60% of the capillary volume [107, 174]. Other major differences between native LC and CZE are listed in **Table 1**.

Hyphenation of CZE with ESI-MS is technically less straightforward and requires a dedicated interface, as both CZE and ESI need closed electrical circuits to function. Currently, two types of CZE-MS interfaces are commercially available: the so-called sheath liquid and sheathless interfaces. The sheath liquid approach employs an additional liquid that ensures electrical contact with the BGE at the outlet of the separation capillary, facilitating both protein CZE and ESI process [175]. This way of interfacing is relatively robust, easy to implement and offers flexibility with respect to tuning protein ionization conditions. Using aqueous solutions of ammonium formate or acetate as sheath liquids will ensure that the HOS of proteins is maintained during ionization, allowing nMS detection [110, 176]. In some cases, use of a denaturing sheath liquid comprising an acid (e.g., formic acid) and/or an organic solvent (for example, acetonitrile or isopropanol) can be advantageous, as it may aid elucidating the composition of protein complexes and aggregates [177-179]. A drawback of sheath liquid interfacing is that the protein bands exiting the capillary are significantly diluted by the sheath liquid flow (1-5 µl/min), resulting in a loss of MS sensitivity [104]. A recently introduced electrokinetically pumped sheath liquid nanospray interface [107, 110, 180, 181] provides sheath liquid at nl/min flow rates, which allows the application of nano-ESI and, therefore, largely overcomes the sensitivity issues. The sheathless interface, as the name indicates, does not require an additional liquid flow, which allows direct coupling of native CZE to MS. The electrical circuit is closed by a porous tip of the CZE capillary that provides conductivity before the CZE effluent leaves the capillary, resulting in ionization of analyte proteins from the BGE and, thus, nMS conditions [72, 80, 104, 182-184]. Due to the characteristic low flows of CZE (nl/min), sheathless CZE-MS is traditionally hyphenated using nano-ESI, resulting in smaller droplet formation and, thereby, more efficient ionization for native separations [182].

Under native conditions around pH 7, the fused-silica capillary inner surface will be negatively charged, whereas basic proteins (pI > 7) will be net positively charged. This will inevitably lead to their adsorption to the capillary wall, potentially causing serious peak tailing, migration time shifts and/or loss of protein. In order to prevent basic protein adsorption, the inner surface of the capillary can be coated with a positively charged (providing electrostatic repulsion for basic proteins) or neutral chemical agent. Coatings for CZE-MS should be static and stable [185], so that no coating agent leaches to the ESI

source, causing background signals [186]. Coated capillaries are commercially available, but can also be prepared in-house prior to CZE analysis [187]. Positively charged coating agents, such as polybrene (adsorptive) [187] or polyethylenimine (covalently bound) have been extensively used for intact protein separations at low pH (denaturing conditions) by CZE-MS. Native CZE-MS methods often rely on capillaries coated with a neutral (uncharged) agent, such as polyacrylamide or cellulose [80, 179]. Neutrally coated capillaries profit from lower flows (with virtually no electro-osmotic flow) and, therefore, may provide enhanced CZE resolution for structurally similar proteoforms [72, 110, 178, 183, 188].

2.4 Affinity approaches

An essential question arising from the diversity of proteoforms associated with a particular protein species is how, and to what extent, each individual form interacts with receptors or ligands. Conventional analytical methods for affinity or binding assessment techniques usually provide an average response from which the contribution of the different proteoforms cannot be derived. Affinity assessment of an individual proteoform would first require its tedious production/synthesis (if at all possible) or isolation/purification. Involving affinity mechanisms in native protein separations hyphenated with MS offers the opportunity to specifically address binding and structure-function relationships of individual proteoforms from mixtures in a fast and comprehensive way. MS facilitates the characterization of proteoform and proteoform complexes after affinity-based separation, but also provides the selectivity to discriminate between proteoforms exhibiting similar affinity. In the LC format of affinity separation, stationary phases with specific immobilized ligands or receptors are often used, and retention of proteoforms is essentially a function of their binding with the ligand/receptor. In SEC and capillary electrophoresis (CE), affinity-based separations can be obtained by adding a ligand or receptor of interest to the mobile phase or BGE, and affinity constants can be determined by measuring changes in the retention or electrophoretic mobilities of proteoforms as a function of ligand/receptor concentration.

2.4.1 Affinity liquid chromatography-mass spectrometry

Affinity LC is an established technique for protein purification; it can also be used for the determination of protein binding characteristics, including the assessment of enzyme-substrate, antibody-antigen and lectin-glycoprotein interactions [189]. In general, to immobilize a receptor on Sepharose-streptavidin beads, they are incubated with biotinylated receptors [189-191]. This column

material comes typically with a low-pressure limit (around 5 bar) and relatively large average particle size (that is, ~34 µm). Smaller column dimensions (for example, 5 mm inner diameter and 50 mm length) [192, 193] are usually preferred, as less material is required for the packing. In affinity LC, the initial mobile phase composition reflects physiological conditions, allowing specific and relevant binding of target proteins with the immobilized ligands or receptors, while non-binding species are not retained. By gradually disrupting the specific interaction between the protein analyte and stationary phase by steadily changing the pH or ionic strength of the eluent, the proteoforms can be eluted in order of increasing affinity [189].

The concept and potential of affinity LC-MS for the characterization of mAb proteoforms has recently been demonstrated using LC columns holding immobilized fetal/neonatal Fc receptors (FcRn) and FcγRIIIa receptors [192, 194]. The interaction of mAbs with FcRn is pH dependent: binding occurs at slightly acidic pH (5.5-6.0) in the endosome, whereas dissociation occurs at pH 7.4 in the blood [191, 195]. Therefore, for FcRn-LC-MS of mAbs, pH gradient elution (a mobile phase of 150 mM ammonium acetate of pH 5.5-8.8) was very useful [191, 194, 196]. In a similar fashion, FcγRIIIa-LC-MS could distinguish and characterize glycoforms of therapeutic mAbs based on their FcγRIIIa affinity. In this case, the interaction between mAbs and the FcγRIIIa receptor was disrupted by applying a decreasing pH gradient (50 mM ammonium acetate, pH 5.0-3.0) [192]. Both examples show how affinity LC-MS can aid our understanding of individual proteoform structure-function relationships, which, for example, is highly relevant when assessing critical quality attributes related to mAbs. Notably, relatively large amounts of protein sample (≥ 50 µg) were needed for the affinity LC-MS analyses. Moreover, the availability of adequate affinity LC phases is still very limited, which is partly related to the large receptor amounts that are required to produce the affinity columns. These obstacles may be mitigated by miniaturization of the affinity columns (by decreasing the column inner diameter), which would entail much less sample and receptor material, while enhancing MS sensitivity by the use of lower flow rates.

In order to avoid problems arising from immobilization of receptors or ligands on stationary phase materials to prepare affinity columns, so-called kinetic SEC-MS has been explored to probe interactions between proteins and low-molecular-weight ligands [197-200]. The protein receptor and ligand are injected on the SEC column after pre-incubation or by sequential injection of the protein and the ligand [199, 200]. The continuous dynamic equilibrium between protein and ligand, and simultaneous SEC separation of protein and ligand, results in a convoluted chromatogram, the shape of which depends

on the specific k_{on} and k_{off} values. These rate constants can be found by deconvoluting the obtained retention pattern using computational fitting [197, 198]. By employing aqueous mobile phases containing ammonium acetate or formate, MS-compatible native conditions were achieved. These approaches are useful to measure protein affinities of, for example, (unknown) inhibitors and metabolites using conventional SEC columns. Notably, the MS selectivity was mainly applied to distinguish ligands, and not to characterize different proteoforms. Another recent native SEC approach proposed using surface plasmon resonance and MS detection in parallel, employing a post-column split. This setup permitted the assignment of protein pharmaceutical size variants by MS while monitoring their individual affinity to the receptor by surface plasmon resonance [144].

2.4.2 Affinity capillary electrophoresis-mass spectrometry

Affinity CE-MS (ACE-MS) is a promising tool to study protein interactions in a proteoform-resolved fashion. Here, the binding receptor or ligand of interest is added to either the sample or BGE, rather than being immobilized on a stationary phase as in conventional affinity LC [201, 202]. Injected proteoforms interacting with the free receptor or ligand during CE analysis exhibit a change in their electrophoretic mobility due to the difference in charge-to-size ratio of the protein complex as compared with the unbound protein. The observed mobility shift is a function of the affinity constant (K_d), which, in principle, can be determined for each of the separated proteoforms. A major advantage of ACE over affinity LC is the possibility to screen a variety of receptors in a straightforward manner, as production of receptor-bound stationary phases is not required. Furthermore, as the ligand and receptor are not immobilized but freely present in the BGE, the risk of altering binding properties because of the immobilization is avoided, yielding true binding parameters [202].

The combination of ACE with MS is particularly beneficial, as, next to obtaining information on proteoform identity, it permits affinity determination of unseparated proteoforms and provides data on binding stoichiometry (**Fig. 4**). Hyphenation of ACE with MS brings some technical considerations and challenges. Binding assays are generally performed using non-volatile buffers, such as sodium phosphate [203]. The use of MS-compatible BGEs may influence protein association-dissociation equilibria and, therefore, buffer effects should be monitored [203]. Fortunately, ammonium acetate or bicarbonate BGEs at physiological pH do not seem to alter the protein-ligand interactions significantly [204, 205]. The capillary should be thermostated to allow for proper K_d determination [206]. To date, ACE-MS has been performed using the sheath

liquid interface. Sheath liquids may influence protein complex stability during the ionization and its composition should be carefully selected to assure native CE-MS conditions. ACE-MS methodologies have been employed to study various protein interactions, including enzymatic inhibition [204, 207, 208], oligosaccharides binding [209] and metabolites [210]. In addition, the combination of ACE with IM-MS is a very powerful approach to reveal binding affinity and heterogeneity, but also the impact of protein conformation on protein activity [208].

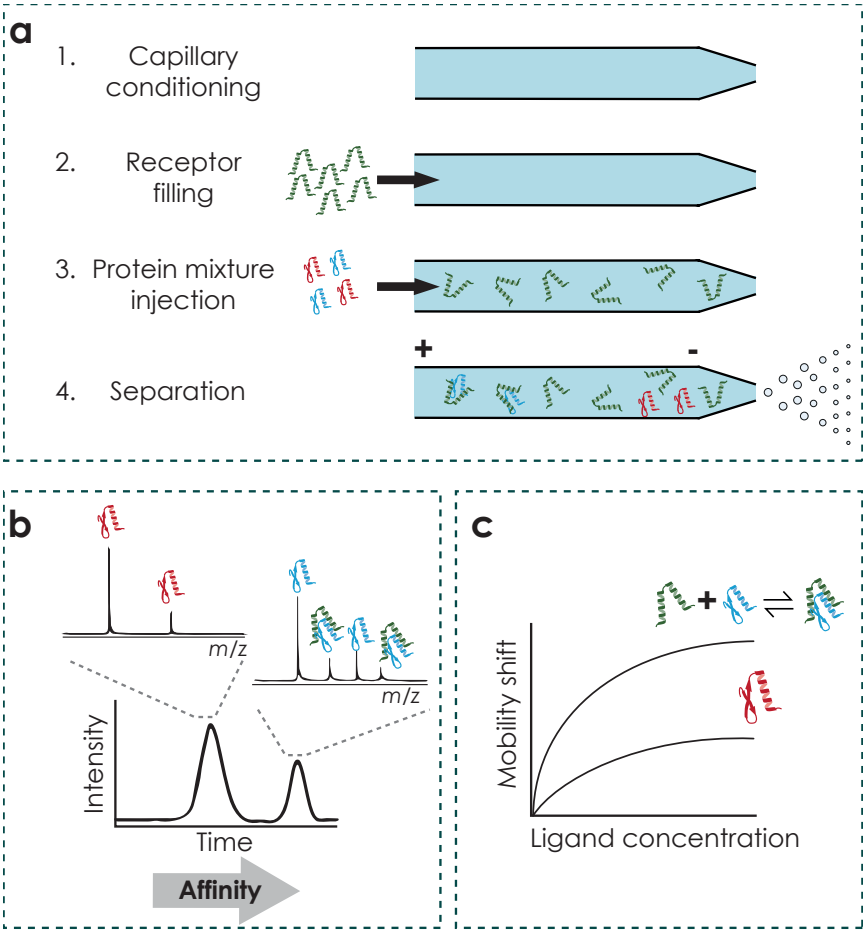


Figure 4 | Schematic representation of the concept of affinity capillary electrophoresis coupled to mass spectrometry. (**a**) Different steps of the analysis with affinity capillary electrophoresis, that is, capillary conditioning, receptor filling, protein mixture injection and separation, followed by online mass spectrometry detection. (**b**) Schematic representation of an affinity capillary electrophoresis separation together with the obtained mass spectra. Proteins interacting with the receptor will exhibit a change in the effective electrophoretic mobility compared with non-interacting species. In this example, interacting species migrate later in the electropherogram. (**c**) The electrophoretic mobility shift obtained with different concentrations of receptor can be employed to obtain binding curves and determine equilibrium constants. Part b adapted with permission from [205].

There are three different modes of ACE that depend on the binding kinetics of the studied protein-ligand interactions (which are dynamic equilibrium, pre-equilibrated and kinetic) [202, 211]. A detailed description of the ACE modes can be found elsewhere [212]. Each of these ACE modes has been combined with MS [204, 207-210, 213]. While for pre-equilibrated mixtures and kinetic approaches the receptor is only present in the injected sample, in dynamic equilibrium ACE, the ligand or receptor is added to the BGE. For the latter, the added ligand/receptor is continuously fed into the MS source, potentially causing ionization suppression (especially for large protein receptors). Recent studies show that protein-protein affinities at the micromolar range or lower may be accurately measured without seriously affecting signal intensity [204]. For weaker affinities or large protein receptors, strategies such as partial-filling ACE could be used to avoid the adverse effects of receptor in the MS source [212].

2.5 State of the art of native separation - mass spectrometry

So far, the application of most native separation-MS methods has focused on the characterization of biopharmaceutical proteins, but there is a growing interest to use the involved technologies for solving clinically related questions. Major questions requiring native analysis of protein samples are related to the presence of potential immunogenic aggregates, unfolded species and diverse conformations.

During production, downstream processing or storage of mAbs, various post-translational modifications can be introduced or lost, potentially leading to reduced efficacy or undesired immune responses. Monitoring these changes is essential to ensure product quality and, thereby, patient safety. For this purpose, an analytical toolbox is available, of which native separations coupled to MS are essential for detection of non-covalent complexes and functional characterization of proteoforms. Both CEX-MS and SEC-MS are already widely applied in this respect. Various CEX-MS methodologies using (salt-mediated) pH gradients have been developed for the identification of charge variants of mAbs, including adalimumab, bevacizumab, cetuximab, infliximab and trastuzumab [115, 157-159, 161, 163, 164]. A variety of proteoforms could be identified, including forms with lysine truncation, glycation and lower abundant modifications (for example, aspartic acid isomerization and deamidation). SEC-MS is mostly used to study the presence of aggregates (for example, dimers) or fragments (for example, light and heavy chains) of mAbs [73, 111, 145]. These species can either induce serious immunogenic responses or have different pharmacokinetics/potency, highlighting the importance for proper analysis methods. To a lesser extent, HIC-MS has been

used for the characterization of proteoforms that differ in hydrophobicity due to, for example, oxidation or O-glycosylation [88, 171]. In particular, oxidation of biopharmaceuticals can lead to decreased half-life and altered FcRn receptor binding [56]. The FcRn receptor is involved in several essential processes *in vivo*, including the recycling of antibodies after endocytosis and the transfer of antibodies across the placenta from mother to fetus. Binding to this receptor is specifically hampered by methionine oxidation in the CH2-CH3 domain of mAbs, making it interesting to investigate the behavior of mAbs before and after exposure to different oxidative stress conditions. For this purpose, affinity LC-MS is an excellent technique, as oxidized forms have lower retention times compared with the non-oxidized protein, indicating decreased affinities for the receptor [194]. FcγRIIIa is another receptor of high pharmacological relevance, as it has a key role in mediating antibody-dependent, cell-mediated cytotoxicity. The interaction of (therapeutic) mAbs with the FcγRIIIa receptor is greatly affected by the Fc glycosylation, highlighting the importance to determine the affinity in a glycoform-specific manner. The retention times of glycoforms depend on fucosylation, with higher affinity for afucosylated glycoforms. The use of online MS detection permits the study of binding of low-abundance proteoforms and/or proteoforms with small differences in affinity, such as galactosylation or bisection, which cannot be addressed with UV detection. This level of proteoform-resolved affinity is often hard to achieve with conventional binding techniques due to interferences between the different glycosylation features (even with glycoengineered antibodies) [192].

ADCs have also been extensively studied using native separation techniques. ADCs contain cytotoxic drugs linked via cleavable linkers to either lysine or cysteine residues of the protein backbone. The potency and safety of these ADCs are directly related to the number of drugs attached to the mAb, making it essential to develop reliable (native) characterization methods. Especially for cysteine conjugate ADCs, denaturing LC-MS-based approaches cannot be used, as the information on the drug-to-antibody ratio (DAR) is lost. To this end, HIC is considered the golden standard for the characterization of (cysteine-linked) ADCs due to the native separation conditions and high separation power for these species, including isoforms with the same DAR attached at different sites [88, 100, 112]. SEC-MS has recently been proposed as an alternative to HIC for quantitating the DAR of ADCs since the online hyphenation of SEC and MS is more straightforward than for HIC [97, 214]. The quantification of DAR by SEC-MS showed no bias and SEC-MS data can be easily bridged to HIC-UV data [214]. Of note, SEC is employed for desalting purposes prior to MS detection and, as a result, all variants elute in

one peak. Therefore, differentiation of positional isomers is not possible using this approach.

Aside from mAbs, native strategies can provide valuable information on other biotherapeutic proteins. For instance, the in-depth characterization of erythropoietin was performed with a pH gradient AEX-MS, which lead to the identification of several critical quality attributes (number of sialic acids and N-acetyllactosamine units), together with other modifications (deamidation and O- acetylation of sialic acids) [101]. In addition, CZE-MS was able to separate different conformers and heterodimers of antithrombin, a biopharmaceutical product used for treatment of hereditary or severe acquired antithrombin deficiencies. This is particularly important as only the native form shows activity, which makes CZE-MS a valuable tool to study the interconversion of these species and to monitor the variants in AT preparations [75]. Finally, structural characterization of other biotechnological proteins, including industrially produced enzymes, may also benefit from the native separation approaches hyphenated to MS. For instance, a prolyl-alanyl-specific protease used for proteomics applications was analyzed with AEX, followed by identification with online MS detection, as well as fraction collection for functional assignment. Proteoforms with a different level of phosphorylated glycans remained active after AEX separation, permitting their isolation and further study of the specificity in a proteoform-specific manner [215].

Some biomedical applications may also greatly benefit from the use of native separation techniques with MS. For example, β_2-microglobulin (β_2m) is a protein associated with dialysis-related amyloidosis, in which the unfolded versions are a key intermediate prior to amyloid fibril formation. Therefore, to study misfolding of β_2m, AEX-MS and CZE-MS methods have been developed [104, 154]. Overall, the coupling of charge-based separations with MS has proven to be suitable for monitoring conformational equilibria, as the unfolded versions exhibit different exposed charges, permitting their separation prior to MS analysis. In both examples, the interconversion of native β_2m to the (partly) unfolded form was studied by the addition of acetonitrile in the sample and/or mobile phase [104, 154]. Another example is human serum albumin, the most abundant protein in human plasma. Due to its interaction with the FcRn, human serum albumin has a prolonged total half-life of about 12-20 days. During this time, different structural degradations (for example, glycation, oxidation, deamidation and truncation) may occur, potentially leading to altered functionality. As these modifications result in a change in charge, AEX-MS as well as CZE-MS methods were developed. Using AEX-MS, nine different species were separated, including forms with C- or N-terminal truncation,

glycation, deamidation and oxidation (cysteinyl, cysteinylglycinyl and sulfinic forms) [155]. Using CZE-UV, a similar separation was obtained compared with AEX (also nine different peaks separated), while coupling MS resulted in poor separation (one peak containing all proteoforms). Nevertheless, using the obtained mass spectrum, the detected proteoforms with AEX-MS could be confirmed and various additional glycated variants were identified.

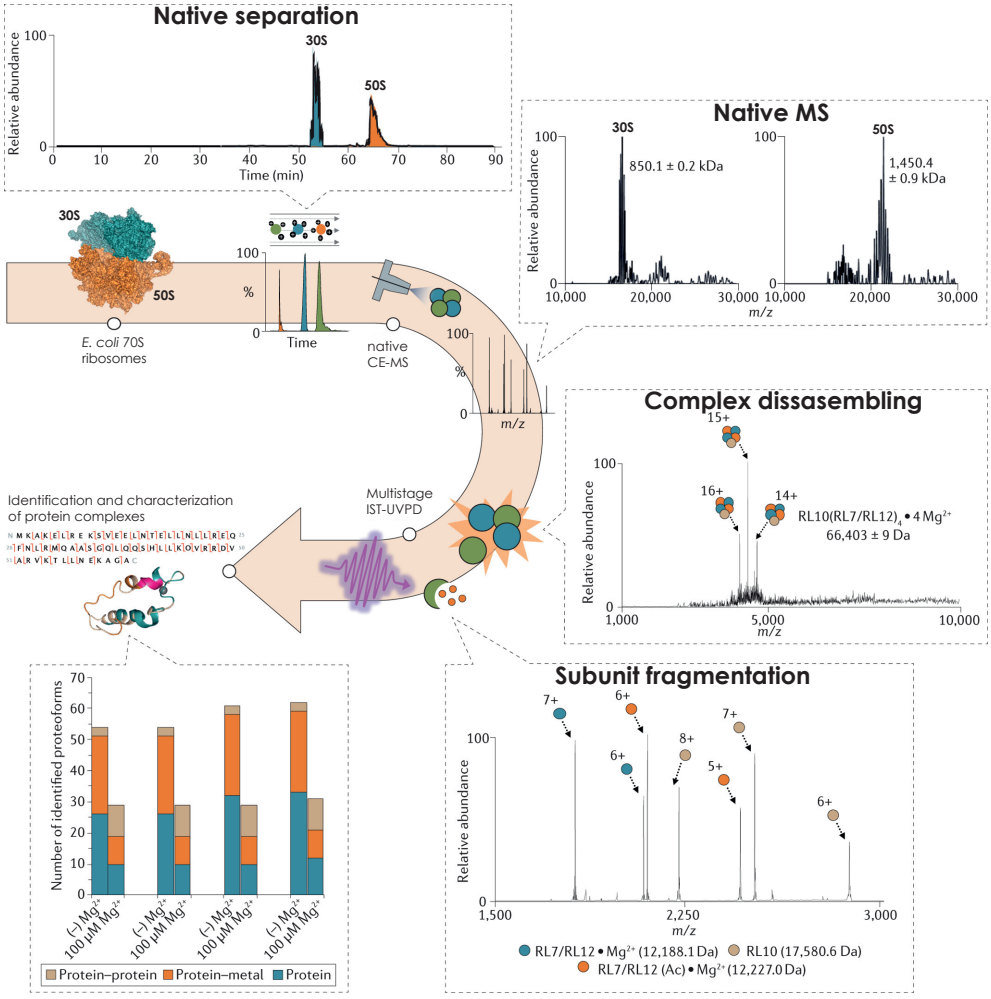


Figure 5 | Example of top-down native CE hyphenated with MS for the characterization of Escherichia coli ribosomal proteins and protein complexes. CE, specifically CZE, can separate 50S and 30S *E. coli* ribosomal subunits and native MS provides molecular mass determination of the subunits. A multistage MS/MS approach consisting of front-end collisional activation (to disassemble the protein complexes into constituent subunits, also called in- source trapping (IST)) and subsequent isolation and activation by high-energy collision density (HCD) or ultraviolet photodissociation (UVPD) (to provide suitable sequence coverage for the identification and characterization of *E. coli* ribosomal proteins). Adapted with permission from [110].

Currently, most native separation-MS applications deal with the characterization of proteoforms of target proteins. In the expanding field of native top-down proteomics, researchers need high-resolution native separations to resolve the vast heterogeneity of the native proteome. In this regard, native CZE-MS is receiving significant attention due to its straightforward native compatibility, high resolution, nanoflow and size dynamic [174, 216]. Native top-down CZE-MS is useful to characterize metalloenzymes, *Escherichia coli* ribosomal complexes and the mononucleosome [80, 110, 176, 183], and to examine metal cofactor binding in ribosomal proteins [110]. Information on the intact structure of the *Escherichia coli* 70S ribosome, subcomplexes and single proteins was obtained by varying the Mg^{2+} concentration in the BGE (100-500 µM) and measuring with and without the ribosomal RNA (**Fig. 5**). This approach allowed the identification of 48 *Escherichia coli* ribosomal proteins, of which several were protein-metal and protein-protein complexes [110]. In addition, native top-down CZE-MS has been applied to analyze the complete proteome of *Escherichia coli* [176]. To achieve this, prefractionation with native SEC was applied to reduce the complexity. Concurrent analysis of these fractions by CZE-MS resulted in the identification of 144 proteins, 672 proteoforms and 23 protein complexes [176].

2.6 Conclusion

The hyphenation of native protein separations with MS has emerged as a useful tool for the structural and functional characterization of proteins. While most of the pioneering and current works focused on methodological developments, a shift towards more application-driven studies can now be observed. Native SEC-MS, IEX-MS and CZE-MS are relatively more established techniques, whereas native HIC-MS still requires further development. The toolbox of native separation approaches is likely to expand with alternative techniques, such as field-flow fractionation and native capillary isoelectric focusing. While hyphenation to MS of these techniques has not yet been reported, we foresee more research in these directions in the near future.

Native top-down MS shows a tremendous growth in the last few years. We expect native top-down proteomics applications to increase in complexity, thereby, challenging the possibilities of both native separations and nMS. This will require further developments in native separation technologies with higher protein resolution and higher peak capacity, while still providing MS compatibility and good sensitivity. A trend towards miniaturized systems, smaller chromatographic particle sizes and new stationary phases is anticipated. In particular, the development of specific native stationary phase materials

enabling higher MS compatibility (by reducing the need for salts in the mobile phase) will expectedly boost the further development of native separation-MS. Separation techniques with prominent MS incompatibility issues may benefit from the increased availability of commercial, multidimensional LC systems, permitting their indirect online hyphenation with MS.

Affinity-based separations coupled to MS have already demonstrated good potential for monitoring interactions of individual proteoforms. Here lies the most relevant, rewarding and challenging purpose we foresee for hyphenated native protein separation-MS techniques in the coming decade: the proteoform-specific assessment of structure-function relationships. When requiring precious affinity materials and/or when sample volumes are very limited, further miniaturization of native separation systems will also be a likely trend and help expand the application of MS-based native protein analysis.

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