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Micro-flow size-exclusion chromatography for enhanced native mass spectrometry of proteins and protein complexes

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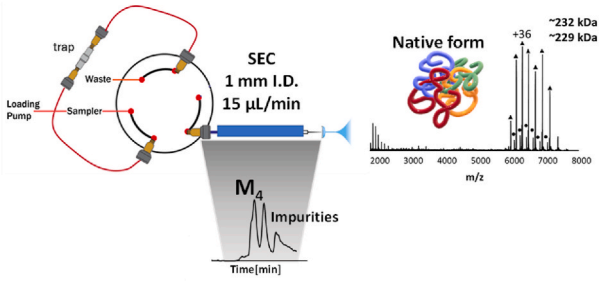
HIGHLIGHTS

- Micro-flow SEC-native MS offers enhanced sensitivity for the detection of higher-order structures.
- Low flow rates (≤15 μL/min) allow milder ionization conditions ensuring native MS of proteins.
- Micro-flow SEC allows the use of higher concentration of volatile salts, preventing protein adsorption more efficiently.
- Trapping the protein before SEC-native MS eliminates the adverse chromatographic effects of large volume injections.

GRAPHICAL ABSTRACT



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ABSTRACT

Size-exclusion chromatography (SEC) employing aqueous mobile phases with volatile salts at neutral pH combined with native mass spectrometry (nMS) is a valuable tool to characterize proteins and protein aggregates in their native state. However, the liquid-phase conditions (high salt concentrations) frequently used in SEC-nMS hinder the analysis of labile protein complexes in the gas phase, necessitating higher desolvation-gas flow and source temperature, leading to protein fragmentation/dissociation. To overcome this issue, we investigated narrow SEC columns (1.0 mm internal diameter, I.D.) operated at 15-μL/min flow rates and their coupling to nMS for the characterization of proteins, protein complexes and higher-order structures (HOS). The reduced flow rate resulted in a significant increase in the protein-ionization efficiency, facilitating the detection of low-abundant impurities and HOS up to 230 kDa (i.e., the upper limit of the Orbitrap-MS instrument used). More-efficient solvent evaporation and lower desolvation energies allowed for softer ionization conditions (e.g., lower gas temperatures), ensuring little or no structural alterations of proteins and their HOS during transfer into the gas phase. Furthermore, ionization suppression by eluent salts was decreased, permitting the use of volatile-

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salt concentrations up to 400 mM. Band broadening and loss of resolution resulting from the introduction of injection volumes exceeding 3% of the column volume could be circumvented by incorporating an online trap-column containing a mixed-bed ion-exchange (IEX) material. The online IEX-based solid-phase extraction (SPE) or "trap-and-elute" set-up provided on-column focusing (sample preconcentration). This allowed the injection of large sample volumes on the 1-mm I.D. SEC column without compromising the separation. The enhanced sensitivity attained by the micro-flow SEC-MS, along with the on-column focusing achieved by the IEX pre-column, provided picogram detection limits for proteins.

1. Introduction

Separation techniques hyphenated with mass spectrometry (MS) and native MS (nMS) are among the primary tools for the structural characterization of biotechnologically-produced proteins [1]. Native conditions (and native analysis methods) maintain the structure, conformation, and higher-order structures of proteins. Advances in MS instrumentation and interfacing options have led the hyphenation of native separations with MS to emerge and bloom rapidly [2,3]. This synergistic combination can support a more-detailed analysis of the higher-order structure (HOS) of proteins and product-related aggregates and impurities. Size-exclusion chromatography (SEC), ion-exchange chromatography (IEX), hydrophobic interaction chromatography (HIC), and capillary zone electrophoresis (CZE) are suitable for the characterization of therapeutic proteins and industrial enzymes under near-native conditions [1]. If conducted properly and after careful optimization (of solvents, buffers, electrolytes, temperature), these techniques allow the preservation of the structural integrity of the protein HOS and the functional native form during the analysis. This is pivotal to establish meaningful structure-function relationships and to draw reliable conclusions on the in-solution state of the proteins, especially in the characterization of labile non-covalent protein complexes and aggregates [4–6].

Among the different chromatographic techniques, SEC is particularly useful for the separation and quantification of protein size variants, such as aggregates, fragments, and impurities of lower molecular weight. In-depth characterization of such size variants is crucial for the quality control of proteins because they may be highly immunogenic and have lower efficacy. The combination of SEC and nMS can bring together the best of the two techniques. The additional separation dimension provided by MS allows distinction of variants that are not separated by SEC [7–10]. SEC separates the sample components according to size prior to their introduction into the MS, and it removes excipients and salts – that may compromise the MS sensitivity – from the sample [8,11,12] [13]. Moreover, the separation of (non-covalent) protein aggregates or oligomeric species may reveal whether such species were present in the analysed solution or were formed or dissociated during the ionization process.

Although SEC coupled to nMS has been successfully reported for applications that are relevant for biopharmaceuticals, such as the characterization of small oligomers and intact antibodies, its use has so far been limited mainly to intrinsically stable biopharmaceuticals [11, 12,14–16]. Despite the indisputable benefits arising from the coupling of SEC with MS, there are some significant practical considerations and limitations. Careful optimization of the separation conditions is required to achieve adequate resolution and to preserve the structural integrity of the proteins and the protein complexes under MS-compatible conditions. The eluent conditions of SEC are critical, as various protein complexes and most aggregates are formed by non-covalent interactions, so that they may disassemble under denaturing conditions. Although SEC is considered a non-retentive chromatographic technique, non-specific chemical interactions can occur between the protein analytes and the stationary phase material, leading to protein adsorption, shifts in elution time, peak asymmetry (tailing) and band broadening [10,17,18]. Additionally, such interactions may induce alterations from the native conformational and functional state of the analysed proteins. To avoid surface interactions in SEC, mobile phases containing high concentrations of non-volatile salts and buffers are often used. Such eluents are not compatible with MS. Certain volatile salts, such as ammonium acetate or formate at high ionic strength (IS; 100–200 mM), have been shown to sufficiently suppress the interactions between the biomolecules and the packing material, enabling characterization of protein HOS, their variants, aggregates and stable protein complexes [10,19]. However, using eluents containing such high concentrations of volatile salts leads to significant ion-suppression in MS, compromising the detection of low-abundant and high-molecular-weight species. Moreover, evaporation of the aqueous solvents used in SEC analysis requires high temperatures during sample introduction into the ion source. The higher desolvation-gas flow and source temperature often result in gas-phase events that disrupt the protein HOS [20]. For example, the increasing internal energy of the ions was reported to lead to denaturing of mAbs during SEC-MS [21]. Although SEC coupled to nMS has been successfully reported for applications of biopharmaceutical relevance, such as the characterization of small oligomers and intact antibodies, its use has so far been limited to intrinsically stable biopharmaceuticals [11,12,14–16].

Conventional SEC columns of 7.8 or 4.6 mm inner diameter (I.D) are operated using typical flow-rates between 0.2 and 1.0 mL/min. In most cases, splitting of the SEC effluent is required to reduce the flow-rate entering the MS instrument and to enable the coupling between SEC and MS. The flow rates directed towards the ion source typically are between 100 and 300 μL/min. At such flow-rates of aqueous solvents, a higher desolvation-gas flow and source temperature are necessary to improve solvent evaporation and enhance the nMS data [12]. It has been shown that even a reduction of the flow-rate from 250 to 100 μL/min can result in an increased signal-to-noise ratio in nMS and improved SEC-nMS performance.

The advantages of a lower flow-rate can be rationalized based on the fundamentals of the ESI process. At a lower flow-rate smaller charged droplets are produced during the spraying process [22]. A way to implement low flow-rates is by drastically reducing the I.D. of the SEC column. So far, the use of a capillary SEC column (300 μm I.D.) has been demonstrated for the characterization of intact monoclonal antibodies (mAbs) [23]. However, capillary SEC columns are not yet commercially available, and they present significant manufacturing challenges [24]. Packing SEC phases into small columns may lead to non-homogeneous stationary phases, leading to uneven flow paths and poor column performance [13]. Additionally, while smaller columns offer increased sensitivity there is a fine limitation of the loaded sample amount. Under micro-flow conditions, extra-column volumes become more critical, as these may lead to significant band broadening. The sample capacity is much lower than for larger columns, since a significant reduction of the injection volume is necessary to maintain the chromatographic performance. Note that due to the non-interactive nature of SEC, both band broadening and limited sample loadability cannot be mitigated by trapping analytes on the head of the columns (common practice in e.g. RPLC). However, to achieve reliable detection of low-abundance proteins, aggregates, oligomers and impurities often relatively large sample volumes have to be introduced. Recently the potential benefits and limitations of coupling narrow I.D. SEC columns (i.e., microbore SEC columns of 1 mm I.D., using flow-rates in the μL/min regime), with nMS for the characterization of intact protein HOS and non-covalent

complexes has only recently gained attention due to the commercially available column options [24].

In the present study, we investigate the use of a commercially available 1-mm I.D. SEC columns, operated at micro-flow conditions (15 μL/min), coupled online to nMS, for improved ionization efficiency and structural characterization of protein HOS, labile protein complexes, and low-abundant proteins. , Additionally, we introduced for the analysis of low-abundant protein variants and impurities, a set-up based on on-line SPE following a "trap-and-elute" approach, using an ion-exchange (IEX) precolumn before the micro-flow SEC-MS analysis. The aim of the developed set-up was primarily to mitigate the adverse chromatographic effects of larger injection volumes, enabling analysis and characterization of protein species with picogram detection limits.

2. Materials and methods

2.1. Chemicals and solutions

The chemicals (purity ≥98%) for the mobile phases were purchased from Merck/Sigma Aldrich (Darmstadt, Germany). All solutions were prepared using ultrapure water (resistivity 18.2 MΩ cm) produced with Sartorius Arium 611UV equipment (Göttingen, Germany). The phosphate-based eluent (pH 6.8) comprised 50 mM sodium phosphate dibasic, 50 mM sodium phosphate monobasic, 100 mM sodium sulfate and 1 g/L sodium azide (≥99.5%). For SEC-nMS eluents ammonium acetate (≥98%) was used at concentrations of 20–400 mM at pH 6.8.

Thyroglobulin from bovine, pyruvate kinase from rabbit muscle, γ-globulin from bovine, transferrin from human serum, ovalbumin from chicken, myoglobin from horse, RNase A from bovine pancreas, and uracil were purchased from Merck/Sigma Aldrich. The therapeutic enzyme L-asparaginase (ASNase, Paronal) produced by E. coli was provided by the Department of Pharmaceutical Analysis, Faculty of Pharmaceutical Sciences, Ghent University (Belgium). The monoclonal antibody (mAb) trastuzumab (Herceptin) was from Roche (Basel, Switzerland). Table S1 in Supplementary Material provides information on the molecular weights (MW) and the iso-electric points (pI) of the proteins used in this study.

2.2. Instruments and methods

Chromatographic system. An UltiMate RSLCnano system (Thermo Fisher Scientific, Breda, The Netherlands) was used, equipped with a high-pressure pump with micro-flow selector, a loading pump (NCS-3500RS), two 10-port two-position valves, and an autosampler. A UV absorbance detector (VWD3400RS) set at 280 nm was used for the SEC measurements. Injection loops of 1, 5 and 20 μL were used. An injection loop of 1 μL was used for recording the calibration curve and for the SEC-nMS experiments. The autosampler temperature was 8 °C. For micro-flow SEC experiments, the eluent flow-rate was set at 15 μL/min. A TOSOH TSKgel SuperSW3000 column (Griesheim, Germany; 300 mm × 1.0 mm I.D., 4 μm particle size, 250 Å pore size) was used. A 50-μm frit (VICI, Houston, TX, USA) was installed before the SEC column.

Protein trap columns (40 mm × 0.50 mm I.D.) were prepared in-house using materials from PolyLC (Columbia, MD, USA). Weak-anion exchange (WAX; PolyWAX), and strong-cation exchange (SCX; PolySulfo A) stationary phases were based on fully porous silica (300 Å pore size). The two materials were mixed (0.03 g PolySulfo A + 0.03 g PolyWAX), and slurry-packed into the trap column using 600 μL MeOH/IPA (50:50, v/v)). Most proteins were loaded on the trap in 10 mM ammonium acetate; for trastuzumab, 100 mM ammonium acetate was used. Loading was performed at 15 μL/min for 3 min. Subsequently, the valve was switched to the SEC mobile-phase (400 mM ammonium acetate) and the trapped proteins were directed to the SEC column. A schematic representation of the trap-and-elute set-up is presented in the Supplementary Material, Fig. S1.

Conventional SEC-MS experiments were performed using an Agilent AdvanceBioSEC column (Wilmington, DE, USA; 150 × 4.6 mm, 2.7 μm, 300 Å) operated at a flow-rate of 200 μL/min and using an injection volume of 5 μL.

The SEC columns were prepared for use by flushing and cleaning them according to the vendor's instructions. To switch between volatile and non-volatile salt mobile phases, the columns were flushed with water for at least 5 column volumes. Additionally, the columns were conditioned with the mobile phase for at least 5 column volumes before use. After measurements were taken, the column was flushed with water for 4 column volumes and then stored in 20% methanol.

SEC-MS. A QExactive Plus Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with an ESI source and operated in positive-ion mode was used for the SEC-nMS experiments. The MS instrument was controlled by Xcalibur software version 4.5 (Thermo Fisher Scientific). Mass analysis of the proteins was performed in the 2000–8000 m/z range. The MS conditions wereas follows. Spray voltage, 2.5 kV; capillary temperature, 275 °C; collision-induced dissociation energy (isCID), 20.0 eV; S-Lens RF level 200. The AGC (Automatic Gain Control) target value was 3×10^6, resolution was set to 17,500, and 10 microscans and a maximum injection time of 200 ms were used.

The 4.6-mm I.D. SEC column, operated at 200 μL/min, was coupled to the MS instrument using a 102-μm I.D. emitter capillary. The probe-heater temperature was 175 °C, and the sheath gas was set to 25.00 manufacturer's units. For the coupling of micro-flow SEC to MS, a 76-μm I.D. stainless-steel needle was used. The probe -heater temperature was 80 °C and the sheath gas was set to 15.00 manufacturer's units. The auxiliary gas was kept to 5 units in both cases. To reduce the post-column dead volume during micro-flow SEC, the grounding union of the ESI was bypassed. To ensure safety, the 10-port valve that was connected to the column outlet was grounded as illustrated in Supplementary Material, Fig. S1. Data analysis was performed using FreeStyle 1.6 software (Thermo Fisher Scientific). For deconvolution of protein mass spectra and estimation of the absolute MW, the UniDec program (University of Arizona, Phoenix, AZ, USA) was used [23]. Raw data are available at https://massive.ucsd.edu/ProteoSAFe dataset MassIVE MSV000091582.

3. Results and discussion

3.1. SEC-MS analysis at microliters/minute flow-rate

The objectives of this work were to evaluate the use of 1-mm I.D. SEC columns operated at flow-rates of 15 μL/min to achieve native separation of the protein HOS, and to determine the effect of such micro-flow-rates for enhanced nMS analysis. To this end, the protein bovine serum albumin (BSA), which exists as a mixture of monomers (M), dimers (M_2), and higher-order aggregates (HOA), was analysed using a 1-mm I.D. SEC column (15 μL/min; injected amount, 1 μg; concentration, 15 μM) and a conventional 4.6-mm I.D. column (200 μL/min; injected amount, 20 μg; concentration, 60 μM) using the same mobile phase (50 mM ammonium acetate, pH 6.8). The separation of the BSA species and HOA aggregates in both columns was very comparable. It should be noted that introduction of a flow-rate of 200 μl/min in the MS instrument required the use of a higher desolvation-gas flow and source temperature to enhance solvent evaporation. For the conventional SEC column operated at 200 μL/min the desolvation-gas flow and source temperature had to be raised to 25 manufacturer's units and 175 °C, respectively, whereas for micro-flow SEC-MS 15 manufacturer's units and 80 °C, respectively, were sufficient. In Fig. 1 the results of the analysis of BSA with the conventional SEC-MS and micro-flow SEC-MS are shown. By comparing the MS signal intensities of the BSA monomer (M; 66 kDa) (Fig. 1B) and dimer (M_2; 132 kDa) (Fig. 1C) and their charge-state distributions (CSDs) obtained under the two flow regimes a number of conclusions can be drawn. The use of a 1-mm I.D. SEC column resulted in three or four times higher MS signal intensities for the monomer and dimer,

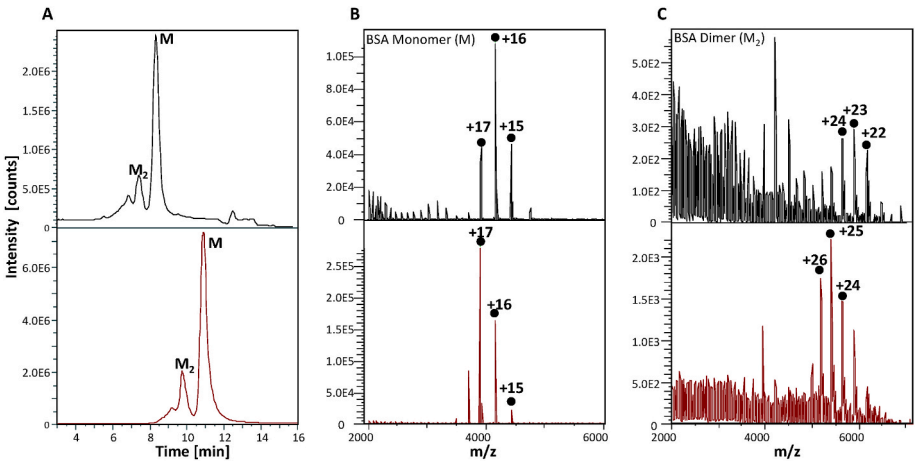

Fig. 1. A) Extracted-ion chromatograms (EICs) of BSA analysed with conventional-flow (top) and micro-flow (bottom) SEC-MS with a mobile phase of 50-mM ammonium-acetate; B) corresponding mass spectra of BSA monomer (M) and C) dimer (M2). Conditions: Conventional-flow SEC-MS: flowrate, 200 μL/min; injected BSA concentration, 60 μM; Micro-flow SEC-MS: flowrate 15 μL/min, injected BSA concentration, 15 μM.

respectively, despite a four-fold lower injected concentration. In the mass spectra of the BSA monomer obtained using the 4.6-mm I.D. column, ions with relatively low *m/z* values (i.e., higher charge states) are observed. At least one additional CSD can be seen. These higher charge states are not observed in the mass spectrum of the BSA monomer obtained using the 1-mm I.D. SEC column. Generally, structurally folded proteins with increased compactness of the polypeptide chain reveal narrower CSDs at higher *m/z* (i.e., lower charge states), whereas denatured and unfolded proteins exhibit broad CSDs including lower *m/z* (i.e., higher charge states) [25,26]. The use of a higher desolvation-gas flow and source temperature to enable solvent evaporation result in an increased internal energy of the ions and this is known to impact the HOS of the proteins, resulting in their denaturation and partial unfolding [27]. it is suspected that BSA undergoes partial unfolding during its transfer to the gas phase. Structural alterations due to interactions between the protein and the column material could also explain this observation. However, such interactions are expected to be more prominent for the diol-type bonded silica 1-mm I.D. column than in the 4.6-mm I.D. column, which features a more bioinert, novel proprietary hydrophilic polymer coated silica packing material [10,28,29]. The chemical properties of the packing material of the SEC columns and the possible residual silanols might give rise to protein denaturation and affects the aggregate recovery and peak shape [10,28,29].

The proteins, ovalbumin, transferrin and trastuzumab were also analysed with the micro-flow and conventional SEC-MS. The injected concentration of the proteins analysed with micro-flow SEC-MS was again four times less than that for conventional SEC-MS. From the distorted peak shapes observed for transferrin and ovalbumin analysed with micro-flow SEC-MS using 50 mM ammonium acetate, protein adsorption and/or interactions with the diol-based column material were suspected (Figs. S2A and B). Higher-ionic-strength conditions (400 mM ammonium acetate) were used to eliminate interactions when using the 1-mm I.D. column. Notably, the conventional column could be operated at low ionic strength because of its proprietary column chemistry that prevents protein interactions [28–30]. Fig. S3 shows the peak areas obtained for BSA, ovalbumin and transferrin when SEC was operated at 400 mM (1 mm I.D. column) and 50 mM (4.6 mm I.D. column) ammonium acetate. Despite the four-times lower concentration introduced in the 1-mm I.D. SEC column, comparable or even higher peak areas (approx. 1.5 times higher) were obtained in comparison with the 4.6-mm I.D. SEC column. Because of the significant gain in sensitivity attained by the reduction of the column dimensions, greatly reduced amounts of protein can be injected on the micro-flow SEC-MS system. These conclusions area in line with the recently published work of Hecht et al. [24], showing that there is an important gain in sensitivity as a result of miniaturization, low-flow rate and reduction of the secondary interactions during micro-flow SEC-MS resulting in possible detection of micromolar affinity complexes.

3.2. *Effect of the salt concentration in the micro-flow SEC-MS*

Using a flow-rate of 15 μL/min of 400-mM ammonium acetate brings approximately 462 μg/min of salt into the ion source (Table S2). This is still 40% less than the amount of ammonium acetate entering the MS instrument when using a conventional SEC column using a flow-rate of 200 μL/min of 50 mM of volatile salt. The latter represents the lowest required concentration of ammonium acetate providing interaction-free SEC using the column with the alternative proprietary chemistry.

To investigate the impact of increasing ionic strength of the mobile phase on the elution behaviour of proteins and the MS signal when using the 1-mm I.D. SEC column operated at 15 μL/min, eluents containing ammonium acetate from 20 up to 400 mM were tested using BSA (Fig. 2). From a comparison of the total-ion chromatograms (TICs) obtained, a shift in retention time is evident. Additionally, a significant loss of resolution between the BSA monomer (M), dimer (M_2) and the HOA is notable when decreasing the ionic strength of the mobile phase. This can be explained by electrostatic repulsion between the negatively charged protein and the deprotonated silanol groups on the stationary phase, resulting in shorter elution times at lower ammonium acetate concentrations. From the results obtained for the SEC-UV analysis of multiple proteins (from ca. 14 up to 660 kDa) using benchmark phosphate-based eluents (pH 6.8; IS > 600 mM) and ammonium acetate (50 up to 400 mM, pH 6.8) eluents, plots of $\log_{10}$MW versus protein elution volume were constructed (Fig. S4). For the PBS eluent (i.e., highest ionic strength), the interactions between proteins and the column material were assumed to be eliminated. Comparison of the elution volumes obtained for the various proteins using the different mobile-phase compositions, revealed that in the range of 200–400 mM ammonium acetate no major shifts in the elution volume of most proteins were observed. A lower IS of ammonium acetate (<200 mM) resulted in shifted elution volumes for nearly all proteins; major shifts were observed for the acidic (BSA, pI = 4.6; ovalbumin, pI = 4.8) and basic (RNAse A, pI = 9) proteins. The concentration range of 200 up to 400 mM ammonium acetate was considered most suitable to suppress unwanted interactions in diol-based SEC columns and improve the recoveries and peak shapes of proteins and aggregates.

Fig. 2B shows the mass spectra obtained for BSA (M) during micro-flow SEC-MS analysis using varying concentrations of ammonium

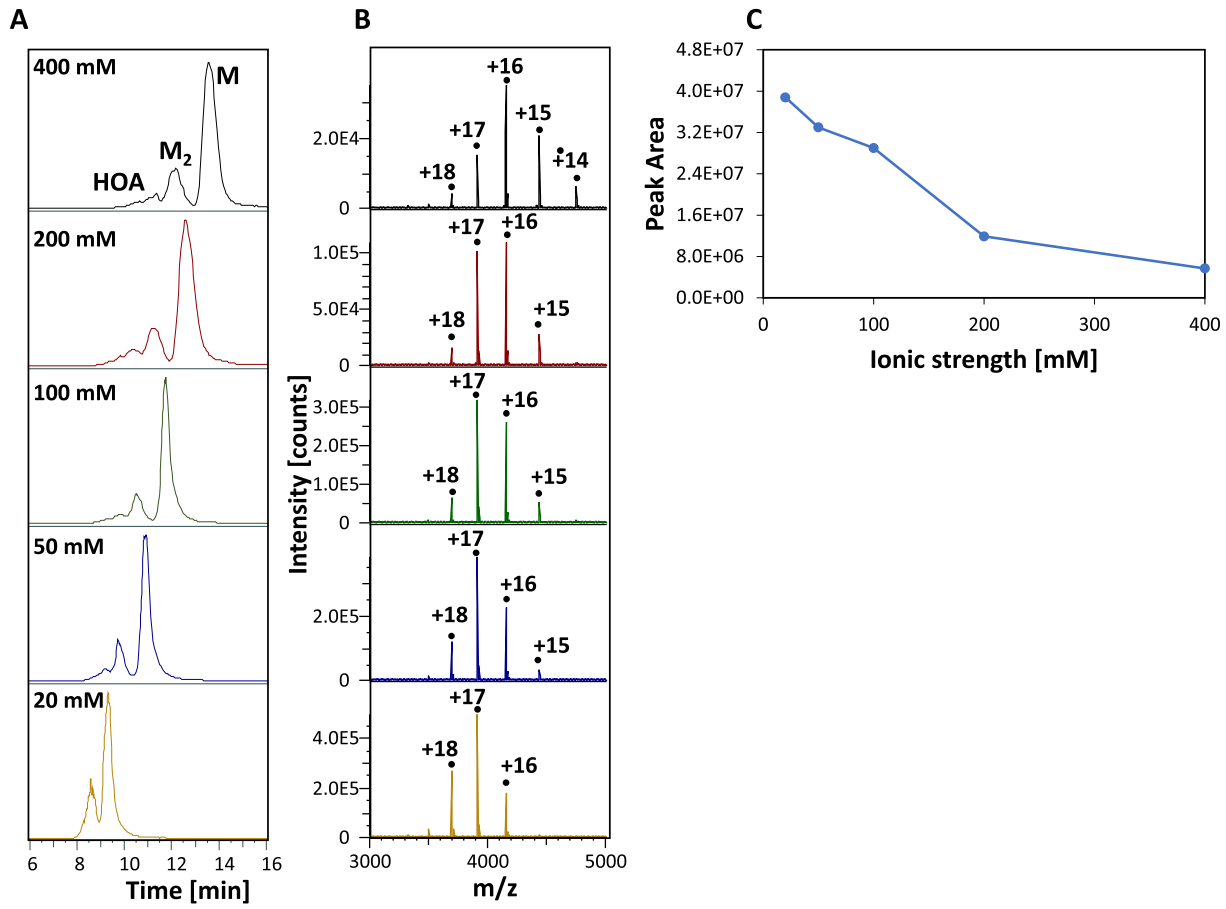

Fig. 2. Micro-flow SEC-MS of BSA (1 μg injected) using eluents containing various concentrations of ammonium acetate at a set flow-rate of 15 μL/min. A) Total-ion chromatograms (TICs) obtained at indicated concentration of ammonium acetate. B) Mass spectra of BSA monomer; C) Peak area of BSA monomer determined from cumulative EICs for the $[M+14H]^{14+}$ to $[M+18H]^{18+}$ charge states (±0.5 amu) versus the eluent concentration of ammonium acetate. The line connecting the experimental points is for visualization only.

acetate (20–400 mM). Lowering the salt concentration of the mobile phase caused a protein-ion peak shift towards lower *m/z* in the mass spectra, i.e., towards higher protein charge states. This shift suggests that BSA (M) may exist in a less compact structure at lower ionic strength [1]. This is in agreement with our previous study, where we found that higher IS (>200 mM) of volatile salts were more likely to ensure preservation of the HOS of the proteins during SEC-MS analysis, due to prevention of the unwanted interactions in SEC or due to gas-phase events during the MS analysis [10,31].

Plotting the peak area of BSA (M) determined from the cumulative extracted-ion chromatograms (EICs) as function of the concentration of the ammonium acetate eluent, showed a clear decrease in the BSA peak area with increasing ionic strength (Fig. 2C). The cumulative EIC was constructed by summing the signal intensities recorded for the $[M+14H]^{14+}$ to $[M+18H]^{18+}$ charge states of BSA (M). Evidently, the high-ionic strength conditions required for near-native SEC, lead to significant protein ionization suppression in ESI-MS. Navigating between the optimal conditions for near-native SEC (i.e., high IS) and the optimal conditions needed for efficient nMS (i.e., low IS) clearly is a challenging task. However, the enhanced MS sensitivity when using micro-flow SEC, seems to outweigh the ionization suppression caused by the high-IS conditions needed for optimal SEC.

Analysis of monoclonal antibodies (mAbs) and non-covalently bound protein complexes and aggregates with SEC can be challenging. For example, careful selection of the SEC conditions is required when analysing relatively basic mAbs (such as trastuzumab and rituximab) and hydrophobic antibody drug-conjugates (ADCs) because of their propensity to adsorb on or interact with silica-based stationary phases. Such interactions may lead to severe underestimation of the amount of HOA [12,28,32]. From the results of the analysis of a degraded trastuzumab sample (6 μM) with micro-flow SEC-nMS at varying concentrations of ammonium acetate (20–400 mM; Fig. S2C) the necessity for careful optimization of the mobile-phase composition is evident. SEC-MS analysis using 400 mM ammonium acetate revealed not only the presence of intact trastuzumab (ca. 150 kDa), but also fragments of ca. 100 and 50 kDa were separated and identified. Lowering the concentration ammonium acetate (<400 mM), resulted in severe deterioration of the separation, with evident protein adsorption (low recovery), shifting elution times, and loss of resolution. Below 100 mM ammonium acetate no protein signal was observed at all, due to complete adsorption of the mAb.

3.3. Monitoring native structures with micro-flow SEC-MS

An important aspect of SEC-MS is to establish whether the (non-covalent) oligomeric species (i.e., dimers, trimers, tetramers) of the proteins and the protein aggregates were actually present in the analysed solution or were formed during the separation and ESI process [1,4,12]. Such structures are often fragile and exist in dynamic equilibria undergoing dissociation or association, depending on the conditions [33]. Therefore, it is essential to ensure that these structures are not disturbed during the SEC-MS analysis. To examine the effect of the flow-rate directed towards the MS instrument on protein-complex structures, we analysed pyruvate kinase (PK) with both micro-flow SEC-MS (15 μL/min; injected concentration 4 μM) and conventional SEC-MS (200 μL/min, injected concentration 17 μM) (Fig. 3). PK is a homo-tetrameric

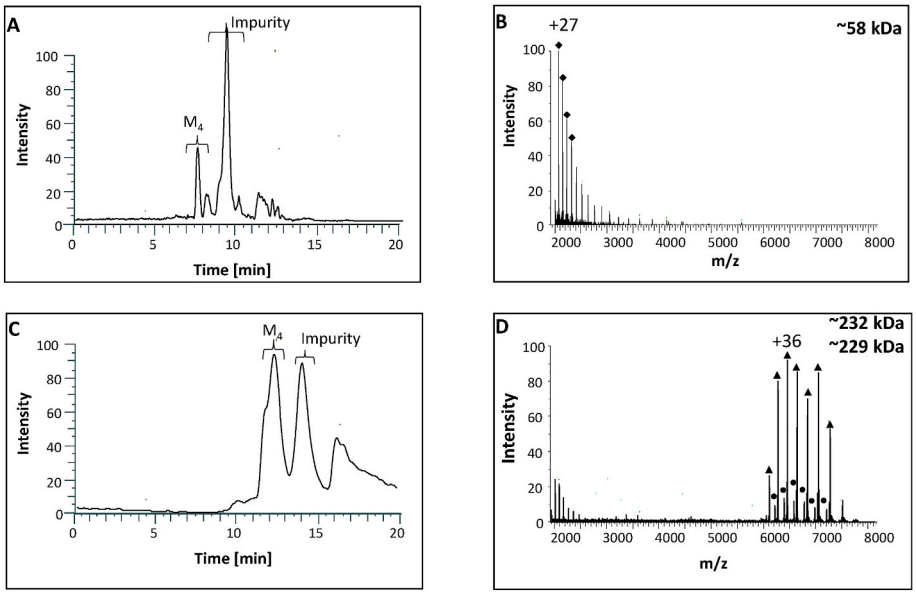

Fig. 3. TICs (A,C) and mass spectra (B,D) obtained during SEC-MS of pyruvate kinase (PK) using A + B) a conventional 4.6 mm I.D. column with a set flow-rate of 200 μL/min, and C + D) a 1 mm I.D. column, with a set flow-rate of 15 μL/min. The mass spectra of the PK tetramer (M4) peak are shown.

(M_4) protein of approximately 230 kDa [34]. For the micro-flow SEC-MS analysis, an eluent of 200 mM ammonium acetate was used, whereas the conventional 4.6 mm I.D. SEC column was operated with 50 mM ammonium acetate. These conditions were considered sufficient for near-ideal SEC analysis for each column. Not unexpectedly, different separation performance was observed for the two columns. As indicated above, the 1-mm I.D. SEC column and the conventional SEC column have different chemistries [10,28,29], as well as different pore sizes (250 Å vs. 300 Å) and particle sizes (4.0 μm vs. 2.7 μm). The required desolvation-gas flow (10 and 20 units) and source temperature (80 and 175 °C) for achieving sufficient solvent evaporation were quite different using 15 μL/min and 200 μL/min, respectively.

Fig. 3A, C presents the TICs obtained for PK analysed with conventional SEC-MS and micro-flow SEC-MS. Based on its molecular weight, M_4 is expected to elute around 7.5 and 12.5 min when using conventional SEC-MS and micro-flow SEC-MS, respectively. An additional peak eluting at approximately 9 min (Fig. 3A) and 14 min (Fig. 3C) was detected in both cases. Its mass spectrum shows no typical protein charge state distribution (Fig. S6). Additionally, the compound appears to not be UV active. The sample component could not be identified, but we presume it is an excipient or impurity. Deconvolution of the mass spectrum obtained for the peak of tetrameric PK using conventional SEC (Fig. 3B), revealed the presence of species with a MW of ca. 58 kDa, which actually corresponds to the monomer of PK. After deconvolution, the mass spectrum corresponding to the tetramer peak of PK obtained with micro-flow SEC-MS (Fig. 3D) confirmed the predominant presence of M_4 species (232 and 229 kDa), with only a minor fraction of monomer being observed in the *m/z* range of 2000–3000. The deconvoluted spectrum revealed two populations of PK tetramers in the sample. The two populations have been previously reported to be generated from the full-length pyruvate kinase (232 kDa) and the truncated PK tetramer (229 kDa) [35]. These observations indicate that the native PK tetramer to a large extent undergoes dissociation in the mass spectrometer under the conditions of conventional SEC-MS, and to a minor extent only when using micro-flow SEC-MS. This is confirmed by the cumulative extracted-ion chromatograms (EIC) constructed for the $[M+27H]^{27+}$, $[M+26H]^{26+}$, $[M+25H]^{25+}$ and $[M+24H]^{24+}$ ions of the PK monomer. Using conventional SEC-MS, the monomer is primarily observed at the position where the tetramer of PK would elute (Fig. S5A), indeed suggesting that native PK is completely dissociating during its transition to the gas phase. Conversely, the cumulative EIC constructed for the $[M_4+35H]^{35+}$, $[M_4+36H]^{36+}$, $[M_4+37H]^{37+}$, $[M_4+38H]^{38+}$ ions observed during micro-flow SEC-MS shows the M_4 signal at the expected retention time (Fig. S5B). The harsher ionization conditions (increased desolvation-gas flow and source temperature) necessary for sufficient solvent evaporation when a flow-rate of 200 μL/min is directed towards the mass spectrometer, apparently have a significant impact on the structural integrity of the non-covalent tetrameric PK complex. The lower flow-rates used with the 1-mm I.D. SEC column permit much softer ionization conditions, thus preserving the HOS of PK.

3.4. Improving loadability in micro-flow SEC

Reducing the column diameter results in a reduced loading capacity, and the relative impact of extra-column band broadening due to the injection volume becomes more critical [23,36,37]. The limited loading capacity may be offset by the enhanced sensitivity achieved when a 1-mm I.D. SEC column is coupled to MS, but the band broadening due to the extra-column volume remains a significant issue [24]. Column overloading and extra-column volumes can cause serious peak distortion (e.g., broadening, tailing, splitting) and reduce the chromatographic resolution. To investigate the capacity of the 1-mm I.D. SEC column, the effects of increased injection volumes as well as absolute injected amounts were studied for BSA (Fig. S7). Even at larger injected amounts (up to 20 μ g) the monomer (M) peak remained well resolved from the dimer peak (M_2) and no significant shift in elution time or peak symmetry was observed (Fig. S7A). However, when increasing the injection volume from 1 to 20 μL (i.e., from approximately 0.5%–10% of the column volume), while keeping the injected mass constant at 5 μg, severe deterioration of the separation performance was observed (Fig. S7B). For SEC analysis a rule of thumb is that the sample volume injected should not exceed 3% of the column volume [17]. This guideline is roughly in line with the observations in Fig. S7B. Injecting 2.5% of the column volume (5 μL) led to minimal additional peak broadening and decrease in resolution.

The analysis of relatively large sample volumes becomes highly relevant for the detection of low-abundance proteins and impurities, variants, aggregates and subunits. For such purposes, a set-up based on on-line SPE, i.e., a so-called “trap-and-elute” approach was explored, aimed primarily to alleviate the adverse chromatographic effects of larger injection volumes. A schematic representation of the set-up is shown in Fig. S1 in the Supplementary Material. A mixed-bed ion-

exchange (IEX) trap column was selected because it allows trapping based on the net charge of the protein molecules and because aqueous solutions and buffers compatible with SEC can be used. Trapping and elution of the proteins can be achieved by tuning the ionic strength and/or the pH of the eluent. Avoiding the use of organic solvents is especially important to prevent denaturation of the protein and changes in the HOS. The mixed-bed IEX material may allow efficient trapping of basic and acidic protein species. Trapping of the injected proteins on the trap column was achieved using a relatively low-ionic-strength solution (e.g., 20 mM ammonium acetate). For the elution of the trapped proteins an eluent of higher ionic strength (400 mM ammonium acetate) was used.

Feasibility experiments were performed with micro-flow SEC-UV to assess the separation performance upon injection of large sample volumes, with and without the use of the IEX trap. A BSA sample (0.05 mg/mL) containing M, M_2 and HOAs was injected (20 μL) directly onto the 1-mm I.D. SEC column and also analysed using the trap-and-elute set-up (Fig. 4). The chromatographic peak width, plate height, resolution between M and M_2, and recovery demonstrate improved performance when trapping the 20-μL sample prior to the SEC analysis (Table S3).

One of the challenges encountered in the analysis of protein samples is that the structural variants present, such as aggregates, oligomers, and fragments, cover a wide size range of molecular weights and physicochemical properties. For that reason, we aimed to explore the capabilities of the IEX-micro-flow SEC set-up for the analysis of proteins of different sizes and charges. Various proteins and protein complexes were analysed by micro-flow SEC-UV with and without the trap column. Trapping of the various proteins was performed using 100 mM ammonium acetate at pH 6.8 and for the elution 400 mM ammonium acetate was used at the same pH. Table 1 summarizes the results obtained and in Fig. S8 the respective chromatograms of the analysed proteins are presented. Comparison of the measured protein peak widths revealed a strong refocusing of the protein band upon its elution from the IEX trap,

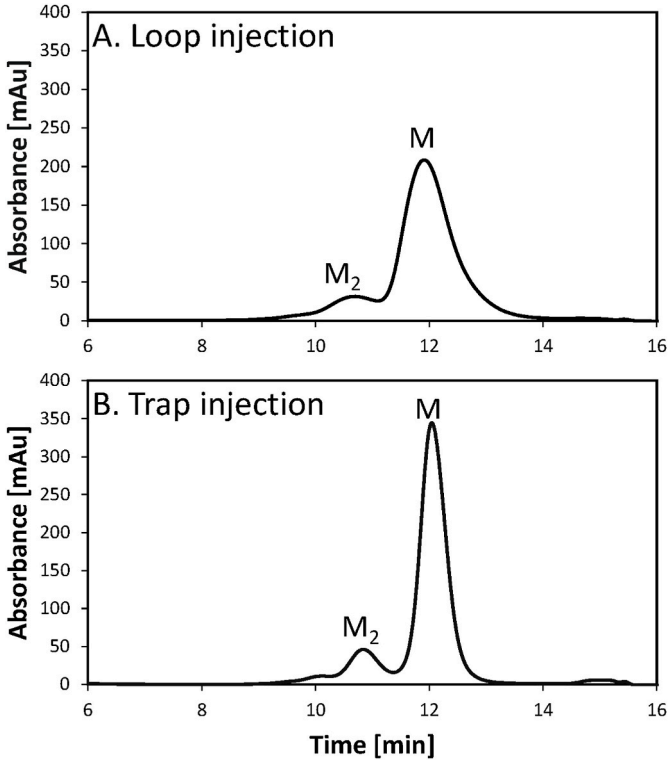

Fig. 4. Micro-flow-SEC-UV of BSA (5 μg) using (A) a 20 μL loop and (B) a 20 μL loop in combination with a mixed-bed IEX trap column for sample injection. BSA was loaded onto the IEX trap column in 20 mM ammonium acetate for 3 min. Elution of the trap column was performed with the SEC eluent (400 mM ammonium acetate). The SEC flow-rate was 15 μL/min.

which significantly improved the SEC performance (reduced band broadening). High recoveries were obtained for most proteins, except for pyruvate kinase. Pyruvate kinase has a pI of 6, which suggests that at the examined pH, the tetrameric protein is only slightly negatively charged. Therefore, it may not be trapped effectively. Protein-specific optimization of the ionic strength and pH of the trapping and elution solvent may be required to ensure high recoveries.

Following the same reasoning, a mixture of four proteins (in total 75 μM) of different sizes (from ca. 14 kDa up to ca. 280 kDa), pIs (the acidic proteins BSA and ovalbumin, and the basic proteins RNAse A and ASNase) and HOS (BSA M M_2 and ASNase M_4 and M_8) was prepared and analysed with the micro-flow trap-SEC-MS set up. Fig. S9 shows the obtained EICs of the respective proteins. All parent proteins and their aggregates (BSA M_2 and ASNase M_8) could be detected with micro-flow SEC-MS following trap-and-elute using the IEX precolumn. The basic proteins, RNAse A and ASNase, seemed to have stronger retention in the IEX trap. As a result, they eluted slower from the trap column and showed longer SEC retention times later than expected. For example, ASNase (M_4, 138 kDa) eluted later than BSA (M_2, 132 kDa) under the examined conditions. Nevertheless, these results indicate that the mixed-bed IEX trap can be a good starting point when dealing with unknown complex mixtures, however, careful optimization of the trapping and separation conditions is essential.

With respect to sensitivity gain, our results show that trapping of the proteins prior to SEC separation provides enrichment as well. Refocusing of the protein analytes on the IEX trap led to reduced band broadening and peak tailing for larger injection volumes (>2.5% column volume). Fig. S10 shows BSA analysed at a concentration of 10 ng/μL (150 nM) using an injection volume of 20 μL, revealing a signal-to-noise ratio of 186 for the $[M+16H]^{16+}$ ion. This corresponds with a total protein load of only 0.2 pg. These results are just an indication of the lowest concentrations that can be analysed with the developed set-up, because different ionization efficiencies are anticipated for different protein species. However, it is evident that the 20-μL injections allowed by the IEX trap, along with the enhanced sensitivity attained by the micro-flow SEC-MS, result in a platform suitable for the characterization of low-abundance proteins, aggregates, and product-related impurities.

4. Concluding remarks

There is a growing need for characterization of proteins and protein biopharmaceuticals in their native state. Their higher-order structures (HOS) need to be preserved during analysis to monitor the presence or the absence of aggregates, oligomers, fragments, conformers, and other product-related impurities. For this purpose, sensitive, non-destructive analytical techniques are required. In this study, we have investigated the potential of a 1-mm I.D. SEC column operated at micro-flow conditions (15 μL/min) coupled online to MS for the characterization of proteins, labile protein complexes and low-abundant protein aggregates and impurities.

The reduction in column diameter and, subsequently, the introduction of low (15 μL/min) flow-rates into the MS instrument provided enhanced sensitivity for proteins. This result is especially important for large protein molecules that are more difficult to ionize and analyse with MS. Micro-flow SEC-MS is very attractive for applications with limited amounts of sample available, or for the detection of low-abundance protein species. Using high-ionic-strength eluents in SEC is important to suppress the unwanted interactions between the analyte and the column material and to ensure preservation of the structural integrity of the protein HOS and the labile (non-covalent) protein complexes and aggregates. The obtained gain in sensitivity by low flow seemed to outweigh the ionization suppression caused by the necessary high-IS conditions. These advantages of micro-flow SEC, along with the mild interfacing conditions (low desolvation-gas flow and source temperature) required, allowed for optimal synergy between interaction-free SEC and nMS. The structural features and HOS of proteins and (non-

Table 1
Peak widths and recoveries for proteins analysed with micro-flow-SEC-UV with and without the trap column. Conditions as in Fig. 3. The relative standard deviation of the recovery (%) is below 7% (n = 3). The relative standard deviation of the peak width measurement difference is below 1% (n = 3).

Protein		MW (kDa)[a]	pI[a]	Peak width (min)		Trap recovery (%)
				No trap	Trap	
Pyruvate kinase (tetramer)		231	6	2.10	1.10	10
L-asparaginase	tetramer	138	8	1.50	0.79	91
	octamer	276		1.49	0.85	91
Trastuzumab		148	8	2.19	1.00	100
BSA		66	5	1.75	0.80	99
Ovalbumin		45	4	2.03	0.93	84

[a] Approximated theoretical values.

covalent) protein aggregates and complexes could be largely maintained during their separation and transfer to the gas phase. This is pivotal in order to gain a better understanding on the structures and species actually present in the analysed solution.

Despite the improved MS sensitivity achieved using low flow-rates, successful analysis of very-low-abundant proteins or highly diluted samples, requires increased injection volumes. Volumetric overloading of the 1-mm SEC column leads to deterioration of the separation performance. Online trapping of proteins using a mixed-bed IEX trap column prior to the SEC analysis was shown to mitigate the adverse effects of large-volume injections. To ensure sufficient trapping of the protein species and high recoveries, protein-specific optimization of the trapping and separation eluents was found necessary. The results of the present study show that the 20 μL injections allowed by the IEX trap, along with the enhanced sensitivity attained by the micro-flow SEC-MS, gave rise to picogram detection limits for proteins.

CRediT authorship contribution statement

Iro K. Ventouri: Conceptualization, Methodology, Investigation, Writing – original draft. **Sharene Veelders:** Investigation. **Marta Passamonti:** Consulted on various aspects of LC instrumentation and supported the coupling of the trap and micro-flow SEC. **Patrick Endres:** Resources. **Regina Roemling:** Resources. **Peter J. Schoenmakers:** Funding acquisition, Supervision, Writing – review & editing. **Govert W. Somsen:** Project administration, Funding acquisition, Supervision, Writing – review & editing. **Rob Haselberg:** Writing – review & editing. **Andrea F.G. Gargano:** Conceptualization, Supervision, Methodology, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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