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Conformation assessment of therapeutic monoclonal antibodies by SEC-MS: unravelling analytical biases for application to quality control

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Abstract:

Immunogenicity related to the degradation of therapeutic monoclonal antibodies (mAbs) remains a major concern for their therapeutic efficacy and safety. Therefore, an analytical method allowing characterization and detection of mAbs degradation is mandatory. In this study, a simultaneous coupling of size exclusion chromatography (SEC) to native mass spectrometry (MS) and fluorescence detection (FLD) is proposed to detect degraded therapeutic mAbs and biases of structural changes (e.g. dimerization, denaturation) that may occur during native MS. A comprehensive study on infliximab behaviors have been performed under different mobile phase conditions (e.g. composition, pH, organic solvent, etc.) and MS parameters (e.g. gas temperatures, CID energies, etc.). Experimental conditions avoiding artificial denaturation and/ or dimerization have been defined. We have also demonstrated that under the developed conditions infliximab affinity towards its biological target TNF α is preserved. In addition, using this method dimers, denatured monomers and fragments could be detected in trastuzumab samples stressed by a long-term storage. These results were confirmed by using SEC coupled to ion mobility mass spectrometry as an orthogonal method for the detection of denatured monomer.

Keywords: therapeutic mAbs, SEC-Native MS, SEC-IMS-MS, artificial dimerization and denaturation, dimer, denatured mAb

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1. Introduction

Quality control of therapeutic monoclonal antibodies (mAbs) in the hospital context plays a pivotal role to ensure their efficacy and safety before administration to patients. Indeed, therapeutic proteins such mAbs could induce immunogenic responses related to antidrug antibodies generated by patients, leading to life threatening situations [1]. Immunogenicity may be provoked by mAbs physical degradations such as unfolding, misfolding and aggregation that occur during their preparation and/or storage [2–5]. Therefore, an accurate method able to detect and characterize these physically degraded mAbs in reconstituted preparations is mandatory. For this purpose, methods allowing detection of conformational/structural modifications and aggregates are required. However, the detection of misfolded and/or weak non-covalent complexes assemblies of therapeutic mAbs are still challenging considering their inherent complexity in terms of size and heterogeneity and their relative instability upon analysis.

Native mass spectrometry (native MS) is nowadays one of the most valuable tool to characterize, non-covalent mAbs interactions and mAbs intrinsically disordered [6–8]. However, when mAbs are physically degraded, coexisting species i.e. folded, misfolded, unfolded or aggregates can be found in the same infusion bag. Thus, mass spectra often show overlapping distributions rendering data interpretation difficult. This is why, coupling of native-MS to a separative method is of particular interest. Among separation methods, size exclusion chromatography (SEC) which is based on differences in hydrodynamic radius, is considered as a reference one to characterize, in complex mixtures, different components such as, high-molecular weight species (HMWS) (i.e dimers, trimers, soluble oligomers), mAbs monomers and low-molecular weight species (LMWS) (i.e mAbs fragments) [9]. Indeed, this method was widely employed for forced degradation studies [5,10] and physicochemical

61 stabilities evaluation of mAbs [4]. In addition, combined to MS, SEC is an online effective
62 way to remove non-volatile molecules by a fast buffer exchange[11].

63 SEC-Native MS or ion mobility spectrometry (IM)-MS methods have recently been
64 reported and employed to characterize antibodies [12–15]. These approaches are able to
65 preserve non-covalent protein-protein interactions [16,17]. Recently, Ehkirch *et al.* has
66 presented an innovative 4-dimensional method to characterize mAbs size variants with SEC x
67 SEC - IM x MS. In this configuration, for the first SEC dimension, a nonvolatile buffer was
68 used for the separation while volatile ones were rather employed in the second dimension to
69 achieve an online desalting [18]. Although this set-up renders SEC fully compatible to MS
70 while maintaining SEC performance, it remains hardly transferable for routine analysis of
71 mAbs infusion bags at the hospital. The approaches frequently employed to render SEC
72 compatible to MS and to limit as much as possible the secondary interactions between
73 proteins and stationary phases employ high concentration of volatile buffers (e.g. ammonium
74 salts) in the mobile phase as well as volatile additives (e.g. organic solvent). However, it is
75 still difficult to prevent HMWS adsorption on the stationary phase [19], [20]. Moreover,
76 artificial protein denaturation, association or oligomer dissociation may occur during the
77 analysis due to ESI native-MS parameters. This renders difficult to draw relevant conclusions
78 from MS data and evaluate the quality of the compounded therapeutic mAbs.

79 To elucidate the impact of analysis conditions and parameters on the modification of
80 protein structure (denaturation, unfolding), mAbs association or aggregate dissociation during
81 SEC-ESI-MS, we developed a simultaneous coupling of SEC to ESI-QTOF and ~~laser-induced~~
82 fluorescence detector (FLD). FLD detecting intrinsic fluorescence of mAbs brings indeed
83 complementary information to MS; (i) molecular weights of different species using a molar
84 mass calibration curve and (ii) molecules undetected by native MS (e.g. very high molecular
85 weight, low amount species). In this work, SEC-FLD/native MS conditions have been also

investigated to reduce mAbs affinity to SEC stationary phases, to ensure high chromatographic performance and high MS sensitivity. The different parameters (chromatographic and MS ones) influencing artificial infliximab oligomers formation have been deeply investigated.

Conditions avoiding analytical biases have been this way defined. We have also checked infliximab ability to interact with its biological target TNF α under the developed conditions. Finally, this SEC method coupled to MS has been applied to stressed trastuzumab sample and revealed evidence of trastuzumab denaturation and dimerization. In addition, analysis of stressed trastuzumab sample by SEC-IMS-MS also allowed to discriminate the denatured versus native conformation of trastuzumab.

2. Experimental section

2.1. Chemicals, Reagents, and Samples.

Ultrapure Water, methanol, iso-propanol, acetonitrile, acetic acid and ammonium hydroxide MS grade were obtained from Merck KGaA (Darmstadt, Germany). Ammonium acetate, ammonium bicarbonate, ammonium formate MS grade, Phosphate buffered saline (PBS) and TNF α were obtained from Sigma-Aldrich (St. Louis, MO, USA). ~~Phosphate buffered saline was purchased~~

Two commercially available mAbs were used: 150mg trastuzumab (Herceptin, Roche, Switzerland) and 100mg infliximab (Inflectra, Pfizer, Belgium).

2.2. Sample preparation.

mAbs lyophilizate were first reconstituted with sterile water for injection to reach the initial concentration required by the manufacturer (infliximab and trastuzumab at 10 mg.mL⁻¹ and 21 mg.mL⁻¹, respectively). Then, each mAbs solutions was separately compounded in 0.9% NaCl according to manufacturer guidelines. Polyolefin FreeFlex® bags for infusion containing 0.9% NaCl were from Fresenius Kabi (France). The final concentration of mAbs solutions were 1.0 mg. mL⁻¹. The compounded mAb solutions were stored at 4° C and protected from light. Compounding, reconstitution and sampling from bags were performed under aseptic conditions using a laminar flow hood.

Fresh sample: After mAbs reconstitution into NaCl 0.9% infusion bag, these samples have been (i) frozen at -20°C during several months; or (ii) stored at 4°C and analyzed within 24 hours. Their mass spectra were similar to those obtained for samples analyzed immediately after their reconstitution.

Stressed sample: Stressed trastuzumab were prepared by storing reconstituted solutions (1.0 mg. mL⁻¹) at 4°C for 6 months (protected from light).

2.3. TNF α -mAb incubation

Commercial TNF α was diluted in PBS buffer at pH 7.3 to obtain a concentration of 0.5 mg. mL⁻¹ (~ 10 μ M). The TNF α solution was subsequently mixed with mAbs (molar ratio ranging from 1.4 /1 to 1/70) in an Eppendorf Protein Lobind tube and stirred (at 300 rpm, 37°C during 30 min) using a Thermomixer C (Eppendorf, Hamburg, Germany).

2.4. Fluorescence Spectroscopy

The intrinsic fluorescence measurements of infliximab were performed with the FP-750 Spectrofluorometer (Jasco Inc, Easton, MD, USA). Excitation wavelength was set at 280 nm and emission has been registered from 295 nm to 450 nm.

2.5. SEC-FLD/MS

SEC-FLD/MS experiments were performed using a 1260 Agilent UPLC, 1260 FLD~~LF~~ detector (Xenon lamp) and ESI-QTOF 6540 mass spectrometer (Agilent Technologies, Santa Clara, CA, USA). An Agilent Bio SEC-3 column (3 μ m, 300 Å, 4.6 x 300 mm) was used. Agilent Mass Hunter B.07.00 software was employed to analyze the MS data. To hyphenate SEC with both ESI-MS and FLD, a post-column splitting flow was set up with a 99:1 (v/v) splitter. The flow rate was set to obtain the same retention time for one given peak using both MS or FLD detection. The mobile phase was pumped at 300 μ L.min⁻¹. FLD detection was performed at λ_{ex} = 280 nm, and λ_{em} 350 nm.

For MS detection, the positive ionization mode was chosen. Nebulizer pressure was set at 25 psig. Transferred capillary, nozzle, fragmentor and skimmer voltages were set at 2500 V, 2000 V, 200 V and 80 V respectively. Profile mass spectra were collected from 1000 to 10000 m/z with an acquisition rate of 0.4 spectrum.s⁻¹.

2.6 SEC-IMS-MS

SEC-IMS-MS experiments were performed on a HPLC Acquity (Waters, Manchester, UK) and a QToF instrument (Synapt G2-Si, Waters Company, Manchester, UK) equipped with a traveling wave ion mobility guide. The column and mobile phase conditions were similar for those for SEC-FLD/MS.

Acquisition of mass spectra were carried out at m/z ranging from 2000 to 14000 and analyzed in the positive ion mode under “near-native” condition. The instrumental parameters were: capillary voltage = 4.5 kV, sampling cone = 150 V, source offset = 150 V, nebulization gas pressure = 5 bar, source temperature = 40 °C, desolvation temperature = 75 °C. The drift gas was nitrogen. T-wave parameters were: Trap gas flow = 2 ml/min; IMS gas flow = 45 ml/min, Trap wave velocity = 300 m/s, Trap wave height = 4 V, He cell gas flow = 120 ml/min, IMS wave velocity = 800 m/s, IMS wave height = 40 V, Transfer wave velocity = 110 m/s and Transfer wave height = 4 V. Calibration was performed using sodium trifluoroacetate. Averaged RMS deviation was around 20 ppm for “native” analyses. After recording ion mobility data, we extracted all information, such as mass to charge ratios (m/z), drift times (t_d) and intensities, using the peak detection procedure of DriftscopeTM software. All the data were gathered in a raw data file (.csv). Then our home-made script allowed extracting information only related to a-Syn* by entering its average molecular mass, the m/z accuracy and limits of the charge states distribution. This allowed us to rapidly process the data and plot $\Omega = f(z)$ (Ω = CCS = collisional cross sections; z = charge state) graphs to reveal the conformational landscape properties.

3. Results and discussion

The impact of the SEC-MS experimental conditions on fresh infliximab (1.0 mg.mL⁻¹) physical degradations (i.e. denaturation, dimerization) was investigated by coupling SEC, with a post-column splitting, to a double detection (FLD and native MS). An optimization of the mobile phase composition has been performed to avoid as much as possible artificial mAbs denaturation, association or aggregate dissociation during infliximab analysis. For native MS, near-physiological conditions as well as soft ionization parameters have been employed to preserve initial conformation and weak noncovalent interactions.

3.1. Optimization of mobile phase buffer for SEC-FLD/MS

3.1.1 Influence of mobile phase buffer electrolytes

We evaluated the influence of the mobile phase buffer at different pHs and ionic strengths on mAbs behavior. At pH 3.0 (acetic acid 100 mM), we can observe by SEC-FLD that the retention time is 6.41 min, whereas at pH 6.0 (ammonium acetate 100 mM), it is increased to 9.27 min (Figure 1A). This t_R shift clearly results from the denaturing effect of acidic mobile phase (pH 3.0) leading to infliximab unfolding and an increase of its hydrodynamic volume. The native-MS detection, at pH 3.0 (acetic acid 100 mM), showed that infliximab fresh sample exhibited a bimodal spectrum with two distinct envelopes. The first envelope of monomers exhibiting very high charge state distribution (CSD) ranging from 29+ to 60+ ions (Figure 1B) confirms the infliximab denaturation which exposes a higher number of charges on its surface. The second envelope has a much lower CSD from 21+ to 29+ ions. According to Rayleigh limit [21], a native form of folded monomer theoretically displays a low charge-density with a narrow CSD with a maximum charge state (Z) estimated at 30+. These results were confirmed by analyzing other therapeutic mAbs (i.e. rituximab, bevacizumab, pembrolizumab, pertuzumab and nivolumab) which also exhibited CDS at 30+ (data not shown).

At near-neutral pH (6.0) and whatever the buffer employed (ammonium acetate, ammonium formate or ammonium bicarbonate) only one peak of monomeric infliximab, exhibiting the same t_R , was observed by SEC-FLD, whereas simultaneous detection by native-MS analysis of this monomeric fraction showed that infliximab analyzed with ammonium acetate (100 mM) exhibited one envelope centered at 28+ corresponding to the folded monomer but also additional one related to dimers formed artificially by MS conditions (Figure 1C). At the same pH with ammonium formate buffer (100 mM), in the monomeric fraction, a partial infliximab unfolding was observed in MS, probably due to a pH transition from pH 6.0 in aqueous phase to pH near pKa 3.7 (formic acid) in the gas phase (Figure 1D). Dimers were also observed with ammonium formate buffer. Concerning ammonium bicarbonate buffer (100 mM, pH 6.0), this tended to fully denature protein during the ionization. Indeed, two envelopes were observed in the monomeric fraction, one with a very high charge state distribution centering at 49+ (unfolded form) and the second with charges centered at 30+ (Figure 1E). Both envelopes could be related to different unfolded or partially unfolded forms. This phenomenon is probably caused by the interaction between proteins in bicarbonate buffer and CO₂ outgassing during ESI leading to bubbles production. Heating and bubbling synergistically induce protein unfolding as explained by Hedge and colleagues [22].

In our SEC-MS experiments, the dimerization phenomenon of infliximab has been observed with most of the mobile phases tested except ammonium bicarbonate (Figure 1 B, C, D). Since ammonium bicarbonate buffer tends to denature proteins, ammonium acetate buffer (100 mM, pH 6.0) has been chosen.

3.1.2. Influence of organic solvents on FLD and MS signal

Organic solvents are often employed in SEC mobile phases to limit the hydrophobic non-specific interaction of protein to stationary phases, and also to enhance the MS ionization efficiency [20]. However, a high percentage of solvent could induce; (i) protein denaturation, (ii) modification of the protein equilibrium between folded and unfolded states, (iii) fluorescent properties modification and (iv) dissociation of non-covalent oligomers. The influence of organic solvents on infliximab fluorescent signal has been first evaluated by adding 10% organic solvent in the compounded infliximab. Both isopropanol (IPA) and acetonitrile (ACN) improved the intrinsic fluorescence intensity of infliximab whereas the signal was reduced with methanol (MeOH) (Figure 2A). In addition, a slight shift of emission maximum (from 345 nm to 337 nm) was observed with MeOH and ACN (Figure 2B). Then, organic solvents (10 %) were added to the mobile phase (ammonium acetate 100 mM pH 6.0). FLD detection showed that infliximab peak area was higher with ACN compared to IPA and MeOH (ACN > IPA > MeOH) (Figure 3), while infliximab peak shape and retention time were similar with the three solvents. With MS detection, IPA addition provided the highest infliximab signal which was approximately 2 and 5-fold higher compared to MeOH and ACN, respectively. Therefore, IPA has been chosen for the following experiments. Most importantly, MS detection showed that a self-association of monomeric mAbs was observed whatever the organic solvent employed.

The influence of IPA % in mobile phase from 5-30% (v/v) has been also evaluated. Retention times, peak shape and peak area remained all constant for infliximab with the FLD detection. On the contrary, in native-MS simultaneous detection, by increasing IPA % (from 5 to 30%) the ionization efficiency of infliximab monomer increased by 1.5-fold while artificial dimerization strongly increases by 2-fold. In addition, possible dissociation of aggregates has been reported by previous studies [23]. Therefore, 10 % has been chosen as a compromised

condition enhancing MS-sensitivity and limiting hydrophobic interactions while avoiding the unwanted mAb conformational changes.

3.1.3. Influence of mobile phase ionic strength, pH and temperature

Ammonium acetate buffers at pH 5.0 to 7.0 with ionic strengths ranging from 60 to 200 mM have been then tested. High pHs and high ionic strengths increase slightly FLD signal while MS signal remained unchanged. Temperature was varied from 20°C to 40°C and no significant difference either on fluorescence or MS signal were observed. Finally, a mobile phase with 150 mM ammonium acetate pH 6.5 has been chosen for mAbs analysis by SEC. FLD detector showed that infliximab exhibits a slight tailing peak ($A_s = 1.3$) in this condition. The method has been validated with infliximab in term of specificity, repeatability (t_R and peak area of the monomer: 0.03 and 0.22 % RSD respectively, $n=6$), intermediate precision (t_R and monomeric infliximab peak area: 0.05 and 0.52 % RSD, respectively, $n=6$). The fractionation domain of this column was found linear from 29 kDa to 669 kDa ($R^2 = 0.9888$).

3.2. Optimization of MS parameters

In the second part of the study, we investigated the ionization and desolvation conditions with the optimized mobile phase to improve the sensitivity and prevent as much as possible mAb structural changes bias. Gas temperatures (sheath gas and drying gas) were found to be the most critical parameters for infliximab ionization. High gas temperatures enhance ionization efficiency and ions transmission compared to low gas temperature. However, in the same time, high temperatures increase the artificial denaturation and dimerization risks during the ionization process. Especially, low sheath gas temperature (i.e. 100°C) combined with high drying gas (i.e. 350°C) could reduce the dimerization rate. In contrast, high sheath gas temperature (i.e. 300°C) with low drying gas temperature (i.e. 150°C) increased the dimerization rate. It could be hypothesized that high sheath gas favors ionization, but low drying gas temperature leads to a poor desolvation and low ions transmission resulting in an

increasing dimerization rate. In-source CID plays also a role in desolvation assist and adduct elimination [24]. We have demonstrated that dimer artefacts were diminished at 25 V and disappeared with collision energy at 100 V (Figure 4). At in-source CID voltage above 100 V, fragmentation of intact infliximab has been observed. Finally, we have chosen 150 °C for sheath gas and 250 °C for drying gas and 100 V for collision energy. When these conditions were employed, no dimerization and denaturation-bias has been observed.

3.3. Interaction TNF α and INF

We also verified that infliximab was maintained under its native state and able to establish interactions with its biological target TNF α during SEC-FLD/MS experiments. The possible monitoring by SEC coupled to FLD of infliximab/TNF α immune complex formation has been previously demonstrated [25]. Therefore, solutions with different ratios of TNF α vs infliximab have been incubated (30 min at 37 °C), keeping constant the concentration of infliximab at 6.7 μ M (1 mg.mL⁻¹) and varying the TNF α ones from 0.1 μ M to 10 μ M. The SEC-FLD-results obtained showed that at the molar ratio of 1/70 (TNF α /infliximab) only one main peak corresponding to monomeric infliximab (t_R 9.2 min) was observed by SEC-FLD (Figure 5A). With the ratio 1/14 two peaks were detected, one monomeric infliximab and a second one that could correspond to a complex TNF α -infliximab. Based on the t_R obtained by SEC-FLD, the molecular weight of this complex could be estimated at 1000 kDa. This large protein complex observed could be explained by cross-linking of several mAbs with TNF α leading to this Infliximab/TNF α complex estimated at 1000 kDa. These results are in agreement with a previous study where the same type of immune complex (Infliximab/TNF α) ranging up to 4000 kDa was observed [26]. At ratio 1/3 four main populations could be observed, with the main peak corresponding to the monomeric infliximab and the three additional ones to different complexes between infliximab and TNF α . By increasing further, the ratio (1/1.4, 1/0.7) all the molecules of infliximab were found to interact with TNF α

suppressing thereby the peak of infliximab monomer. In these latter cases only two different hetero complexes between TNF α and infliximab were evidenced. As the number of different detected hetero-oligomers is varying with the increase of the ratios we can hypothesize that the formation of complexes follows transition states which are TNF α -concentration related. With the obtained results, we can also conclude that the mobile phase containing 10 % IPA in 150 mM acetate ammonium pH 6.0 do not disrupt the non-covalent interaction between TNF α and infliximab. However, these complexes detected by FLD could not be seen by SEC-native-MS using ammonium acetate due to the limited mass range detection of QTOF6450 for the ions above 10 000 m/z . Therefore, in order to confirm the nature of these complexes, the ammonium bicarbonate buffer inducing protein dissociation of non-covalent complexes during MS, has been employed to analyze TNF α -infliximab complexes at ratio (1/3) by SEC-FLD-MS.

FLD detection showed similar retention times and chromatographic profiles between ammonium bicarbonate buffer (pH 6.5 150 mM) and ammonium acetate (pH 6.5 150 mM) buffer. TNF α is an homotrimer eluted at 10.9 min (Figure 5A) that was partially dissociated during the ESI-process. This dissociation has led to the detection of TNF α monomers, dimers, trimers and tetramers by MS spectrum (Figure 5B). The TNF α -infliximab complexes were also dissociated in these MS conditions. In the complex eluted at 6.3 min, we observed high intensities of TNF α monomers and trimers overlapping the denatured infliximab envelop (Figure 5C). The complex which eluted at 7.2 min get dissociated under ESI process releasing mainly denatured INF and a minor signal of TNF α monomer (Figure 5D). These results assessed that mAbs and TNF α are able to interact under the mobile phase conditions optimized for SEC-FLD/MS.

3.4. Analysis of trastuzumab stressed samples

In order to show that this method could be applied to other mAbs, this developed SEC-FLD/MS method has been employed to analyze a stressed sample of trastuzumab (1.0 mg.mL⁻¹) (storage at 4°C during more than 6 months). For the stressed sample, the trimer (t_R 7.2 min), dimer (t_R 7.9 min), monomer (t_R 9.2 min) together with several fragments (t_R 11.0 min, MW~ 50 kDa), were detected by FLD (Figure 6A) while native MS detected only monomers and dimers but not trimers (Figure 6B). In the fresh sample, only one peak of monomer was detected by FLD with a mass spectrum displaying a low CSD centering at 27+ (Figure 6C).

In monomer fraction of the stressed sample eluting from 8.8 to 10.3 min (Figure 6B), the extracted ion current (XIC) showed two peaks (t_R 9.2 and 9.8 min). The extracted-ion chromatogram (XIC) profile of the monomer eluted at 9.2 min exhibited a main envelop with a mass/charge ratio ranging from 5000 m/z to 6200 m/z and charge state centered at 27+ that could correspond to the folded one. This is assessed by the similar charge state displayed by the fresh trastuzumab sample (centered at 27+) (Figure 6C). The XIC profile of the peak eluted at 9.8 min (Figure 6B) exhibited a signal corresponding to a degraded form of the monomer (from 4200 to 5000 m/z) with a deconvoluted mass corresponding to 104170 Da (Figure 6D). The slightly higher t_R of the degraded trastuzumab, could be explained by the smaller molecular weight of the degraded form. Both species folded and degraded forms exhibited a poor chromatographic resolution. The dimer exhibited a mass spectrum ranging from 6000 to 8500 m/z with a deconvoluted mass corresponding to 296 kDa and centered at 41+ (Figure 6E).

To better understand the results obtained by SEC FLD/native-MS, this stressed sample was analyzed by the SEC hyphenated online to ESI-IMS-MS (Synapt G2-Si, Waters, Manchester, UK). For experiments in non-denaturing conditions, instrumental and hardware parameters of IMS-MS were optimized to obtain the best signal to noise ratio for all the protein complexes while preserving intact the mAbs structures. For the stressed Trastuzumab sample, the mass

spectrum for the monomer fraction, showed intact antibody having an average molecular weight of $148\,455 \pm 40$ Da as well as a degraded form with an average molecular weight of $101\,207$ Da (Figure 7A). Since ion mobility data were recorded at the same time, we were able to describe the conformational landscape of the intact antibody (Figure 7B) as well as of the degraded form (Figure 7C). Figure 7B and 7C display the collisional cross sections (CCS) of the molecule as a function of the charge state ($\text{CCS} = f(z)$), called conformational landscape. For non-stressed trastuzumab conformational landscape showed that 3 different conformations (CF1, CF2, CF3) exist in this monomeric fraction. CF3 exhibits a low CCS ($3800\text{--}5000 \text{ \AA}^2$) and low CSD ranging from 26+ to 28+ which corresponds to folded monomer. Reversely, CF1 and CF2 exhibit higher CCS (more than $5000 \text{ \AA}^2$) and higher charges distributions from 28+ to 30+ and 29+ to 31+, respectively for CF2 and CF1 conformers, which correspond to conformers having more expanded structures (Figure 7B). In contrast, the conformational landscape of the degraded form (Figure 7C) showed that trastuzumab cleavage lead to the loss of conformers, that can be related to a lower flexibility.

4. Conclusion

Here we developed a method based on simultaneous coupling of SEC to ESI-QTOF and (LIF) FLD suitable for therapeutic mAbs quality control. This method allows us to clearly identify the experimental conditions leading to artificial conformational changes of mAbs related to MS and to confirm by native-MS the conformational state of the monomeric peak observed in FLD (denatured, degraded or folded). Thus, this set-up allows the discrimination between mAbs dimer artificially formed during the MS analysis from aggregate species originally present in the sample. This method has been successfully applied to trastuzumab stressed sample and allowed detecting degraded forms and dimers present in the sample. An online hyphenation of SEC to IMS-MS confirmed the presence of conformational modification for

363 monomers in trastuzumab stressed samples. This method will allow detection of degraded
364 molecules from mAbs (i.e. dimer, fragments) in infusion bags within a single analysis which
365 ~~is not possible~~
366

Conflict of interest

The authors declare no competing financial interest.

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Figure captions

Figure 1: Online SEC-FLD~~LF~~/native MS analysis of infliximab (1 mg.mL^{-1}) (A) SEC-FLD~~LF~~ chromatogram using 100mM acetic acid pH 3.0 mobile phase (blue trace) and 100mM ammonium acetate pH 6.0 mobile phase (black trace). Mass spectra of monomeric infliximab at different mobile phase (B) 100 mM acetic acid pH 3.0, (C) 100 mM ammonium acetate pH 6.0, (D) 100 mM ammonium formate pH 6.0, (E) 100 mM ammonium bicarbonate pH 6.0.

Column: BioSEC-3, ($4.6 \text{ mm} \times 300 \text{ mm}$, $300 \text{ \AA}$, particle diameter $3 \text{ }\mu\text{m}$); Flow rate: $300 \text{ }\mu\text{L.min}^{-1}$; FLD~~LF~~: $\lambda_{\text{ex}} = 280 \text{ nm}$, $\lambda_{\text{em}} = 350 \text{ nm}$. MS condition: drying gas. $250 \text{ }^{\circ}\text{C} - 6 \text{ L min}^{-1}$, sheath gas $200 \text{ }^{\circ}\text{C} - 6 \text{ L min}^{-1}$, nebulizer pressure 15 psig, transferred capillary, nozzle, fragmentor and skimmer voltages were set at 3500 V, 1500 V, 175 V and 65 V respectively

Figure 2: Fluorescent detection of compounded infliximab with organic solvent (1 mg.mL^{-1}) ; (A) Fluorescence emission spectra, (B) second derivative fluorescence spectra ($\lambda_{\text{ex}} = 280 \text{ nm}$, $\lambda_{\text{em}} = 295 - 450 \text{ nm}$).

Figure 3: Comparison of different organic solvent influence in mobile phase, ACN, MeOH, IPA. Online SEC-FLD~~LF~~/native MS analysis of fresh infliximab with mobile phase containing 10% organic solvent: Mass spectra intensity (orange histogram) and peak area measured by FLD~~LF~~ detector (blue trace) of infliximab monomers. Mobile phase: ammonium acetate 100 mM, pH = 6.0, 10 % organic solvent (v/v), other experimental conditions as mentioned in Figure 1.

Figure 4: Mass spectra of monomeric infliximab fraction at different in-source CID. (A), 25V, (B) 100 V.

Mobile phase: ammonium acetate 150 mM, pH = 6.0 (10% IPA), other experimental conditions as mentioned in Figure 1.

Figure 5: TNF α /infliximab analyzed by SEC-FLDLIF-MS; (A) Chromatograms of TNF α /infliximab mixtures at different ratios obtained by FLD SEC-LIF. Online SEC-MS analysis; Mass spectra of: (B) TNF α , TNF α / infliximab at ratio 1/3; (C) TNF α /infliximab complex at 6.3 7.2 min and (D) TNF α / infliximab complex at 7.2 6.3 min.

M, D, T, Te are monomeric, dimeric, trimeric and tetrameric forms of TNF α , respectively
Mobile phase: ammonium bicarbonate 150 mM pH 6.5 (10% IPA). Column: BioSEC-3, (4.6 mm $\times$ 300 mm, 300 Å, particle diameter 3 μ m); Flow rate: 300 μ L.min⁻¹; FLDLIF: λ_{ex} = 280 nm, λ_{em} = 350 nm.

MS conditions: MS condition: drying gas. 250 °C – 6 L min⁻¹, sheath gas 150 °C – 6L min⁻¹, nebulizer pressure 25 psig, transferred capillary, nozzle, fragmentor and skimmer voltages were set at 2500 V, 2000 V, 300 V and 80 V respectively

Figure 6: On-line SEC-FLDLIF/native MS analysis of stressed trastuzumab (long-term storage (4°C more than 6 months). (A) Fluorescent chromatogram and (B) Total ion current (TIC) of stressed trastuzumab analysis, (C) TIC of monomeric peak (from 9.0 to 10.5 min), (D) XIC profile corresponding to native trastuzumab (t_R = 9.2 min blue line), (E) XIC profile corresponding to denatured degraded trastuzumab of monomer peak (t_R = 9.8 min red line). (C) mass spectrum of fresh trastuzumab (D) Mass spectrum of monomeric fraction of stressed trastuzumab, (E) Mass spectrum of dimeric fraction of stressed trastuzumab.

Experimental conditions as mentioned in Figure 5.

521 ~~Figure 7: On-line SEC-LIF/native MS analysis of trastuzumab (A) mass spectra of fresh~~
522 ~~sample (B) Mass spectra of stressed trastuzumab (t_R 9.2 min in Figure 6D). (C) Mass~~
523 ~~spectrum of stressed trastuzumab (t_R 7.9 min in Figure 6B).~~

524 ~~Experimental conditions as mentioned in Figure 5.~~

525

526 Figure 78: Trastuzumab sample (stressed) analyzed by SEC-IMS-MS (A) Mass spectrum of
527 the monomeric peak (B) Collision cross section of the intact trastuzumab monomer depicted
528 in Figure 87A. (C) Collision cross section of the degraded trastuzumab depicted in Figure 7A.
529 Mobile phase conditions and column as mentioned in Figure 5. IM-MS conditions as
530 mentioned in experimental section.

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