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# 1      **Conformation assessment of therapeutic monoclonal antibodies by SEC-**

## 2      **MS: unravelling analytical biases for application to quality control**

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### 7      **Abstract:**

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

23

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

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36

## 37 **1. Introduction**

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

50 Native mass spectrometry (native MS) is nowadays one of the most valuable tool to  
51 characterize, non-covalent mAbs interactions and mAbs intrinsically disordered [6–8].  
52 However, when mAbs are physically degraded, coexisting species i.e. folded, misfolded,  
53 unfolded or aggregates can be found in the same infusion bag. Thus, mass spectra often show  
54 overlapping distributions rendering data interpretation difficult. This is why, coupling of  
55 native-MS to a separative method is of particular interest. Among separation methods, size  
56 exclusion chromatography (SEC) which is based on differences in hydrodynamic radius, is  
57 considered as a reference one to characterize, in complex mixtures, different components such  
58 as, high–molecular weight species (HMWS) (i.e dimers, trimers, soluble oligomers), mAbs  
59 monomers and low–molecular weight species (LMWS) (i.e mAbs fragments) [9]. Indeed, this  
60 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

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

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

96

97 **2. Experimental section**

98 **2.1. Chemicals, Reagents, and Samples.**

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

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

106 **2.2. Sample preparation.**

107 mAbs lyophilizate were first reconstituted with sterile water for injection to reach the  
108 initial concentration required by the manufacturer (infliximab and trastuzumab at 10 mg.mL<sup>-1</sup>  
109 and 21 mg.mL<sup>-1</sup>, respectively). Then, each mAbs solutions was separately compounded in  
110 0.9% NaCl according to manufacturer guidelines. Polyolefin FreeFlex® bags for infusion  
111 containing 0.9% NaCl were from Fresenius Kabi (France). The final concentration of mAbs  
112 solutions were 1.0 mg. mL<sup>-1</sup>. The compounded mAb solutions were stored at 4° C and  
113 protected from light. Compounding, reconstitution and sampling from bags were performed  
114 under aseptic conditions using a laminar flow hood.

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

119 *Stressed sample:* Stressed trastuzumab were prepared by storing reconstituted solutions (1.0  
120 mg. mL<sup>-1</sup>) at 4°C for 6 months (protected from light).

121

### 122 **2.3. TNF $\alpha$ -mAb incubation**

123 Commercial TNF $\alpha$  was diluted in PBS buffer at pH 7.3 to obtain a concentration of 0.5  
124 mg. mL<sup>-1</sup> (~ 10  $\mu$ M). The TNF $\alpha$  solution was subsequently mixed with mAbs (molar ratio  
125 ranging from 1.4 /1 to 1/70) in an Eppendorf Protein Lobind tube and stirred (at 300 rpm,  
126 37°C during 30 min) using a Thermomixer C (Eppendorf, Hamburg, Germany).

### 127 **2.4. Fluorescence Spectroscopy**

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

### 131 **2.5. SEC-FLD/MS**

132 SEC-FLD/MS experiments were performed using a 1260 Agilent UPLC, 1260 FLD~~LF~~  
133 detector (Xenon lamp) and ESI-QTOF 6540 mass spectrometer (Agilent Technologies, Santa  
134 Clara, CA, USA). An Agilent Bio SEC-3 column (3 $\mu$ m, 300 Å, 4.6 x 300 mm) was used.  
135 Agilent Mass Hunter B.07.00 software was employed to analyze the MS data.

136 To hyphenate SEC with both ESI-MS and FLD, a post-column splitting flow was set up with  
137 a 99:1 (v/v) splitter. The flow rate was set to obtain the same retention time for one given  
138 peak using both MS or FLD detection. The mobile phase was pumped at 300  $\mu$ L.min<sup>-1</sup>. FLD  
139 detection was performed at  $\lambda_{ex}$  = 280 nm, and  $\lambda_{em}$  350 nm.

140 For MS detection, the positive ionization mode was chosen. Nebulizer pressure was set at  
141 25 psig. Transferred capillary, nozzle, fragmentor and skimmer voltages were set at 2500 V,  
142 2000 V, 200 V and 80 V respectively. Profile mass spectra were collected from 1000 to 10000  
143  $m/z$  with an acquisition rate of 0.4 spectrum.s<sup>-1</sup>.

### 144 **2.6 SEC-IMS-MS**



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

149 Acquisition of mass spectra were carried out at  $m/z$  ranging from 2000 to 14000 and  
150 analyzed in the positive ion mode under “near-native” condition. The instrumental parameters  
151 were: capillary voltage = 4.5 kV, sampling cone = 150 V, source offset = 150 V, nebulization  
152 gas pressure = 5 bar, source temperature = 40 °C, desolvation temperature = 75 °C. The drift  
153 gas was nitrogen. T-wave parameters were: Trap gas flow = 2 ml/min; IMS gas flow = 45  
154 ml/min, Trap wave velocity = 300 m/s, Trap wave height = 4 V, He cell gas flow = 120  
155 ml/min, IMS wave velocity = 800 m/s, IMS wave height = 40 V, Transfer wave velocity =  
156 110 m/s and Transfer wave height = 4 V. Calibration was performed using sodium  
157 trifluoroacetate. Averaged RMS deviation was around 20 ppm for “native” analyses. After  
158 recording ion mobility data, we extracted all information, such as mass to charge ratios ( $m/z$ ),  
159 drift times ( $t_d$ ) and intensities, using the peak detection procedure of Driftscope<sup>TM</sup> software.  
160 All the data were gathered in a raw data file (.csv). Then our home-made script allowed  
161 extracting information only related to a-Syn\* by entering its average molecular mass, the  $m/z$   
162 accuracy and limits of the charge states distribution. This allowed us to rapidly process the  
163 data and plot  $\Omega = f(z)$  ( $\Omega$  = CCS = collisional cross sections;  $z$  = charge state) graphs to  
164 reveal the conformational landscape properties.

165

### 166 **3. Results and discussion**

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

#### 174 **3.1. Optimization of mobile phase buffer for SEC-FLD/MS**

##### 175 *3.1.1 Influence of mobile phase buffer electrolytes*

176 We evaluated the influence of the mobile phase buffer at different pHs and ionic  
177 strengths on mAbs behavior. At pH 3.0 (acetic acid 100 mM), we can observe by SEC-FLD  
178 that the retention time is 6.41 min, whereas at pH 6.0 (ammonium acetate 100 mM), it is  
179 increased to 9.27 min (Figure 1A). This  $t_R$  shift clearly results from the denaturing effect of  
180 acidic mobile phase (pH 3.0) leading to infliximab unfolding and an increase of its  
181 hydrodynamic volume. The native-MS detection, at pH 3.0 (acetic acid 100 mM), showed  
182 that infliximab fresh sample exhibited a bimodal spectrum with two distinct envelopes. The  
183 first envelope of monomers exhibiting very high charge state distribution (CSD) ranging from  
184 29+ to 60+ ions (Figure 1B) confirms the infliximab denaturation which exposes a higher  
185 number of charges on its surface. The second envelope has a much lower CSD from 21+ to  
186 29+ ions. According to Rayleigh limit [21], a native form of folded monomer theoretically  
187 displays a low charge-density with a narrow CSD with a maximum charge state (Z) estimated  
188 at 30+. These results were confirmed by analyzing other therapeutic mAbs (i.e. rituximab,  
189 bevacizumab, pembrolizumab, pertuzumab and nivolumab) which also exhibited CDS at 30+  
190 (data not shown).

191 At near-neutral pH (6.0) and whatever the buffer employed (ammonium acetate,  
192 ammonium formate or ammonium bicarbonate) only one peak of monomeric infliximab,  
193 exhibiting the same  $t_R$ , was observed by SEC-FLD, whereas simultaneous detection by native-  
194 MS analysis of this monomeric fraction showed that infliximab analyzed with ammonium  
195 acetate (100 mM) exhibited one envelope centered at 28+ corresponding to the folded  
196 monomer but also additional one related to dimers formed artificially by MS conditions  
197 (Figure 1C). At the same pH with ammonium formate buffer (100 mM), in the monomeric  
198 fraction, a partial infliximab unfolding was observed in MS, probably due to a pH transition  
199 from pH 6.0 in aqueous phase to pH near pKa 3.7 (formic acid) in the gas phase (Figure 1D).  
200 Dimers were also observed with ammonium formate buffer. Concerning ammonium  
201 bicarbonate buffer (100 mM, pH 6.0), this tended to fully denature protein during the  
202 ionization. Indeed, two envelopes were observed in the monomeric fraction, one with a very  
203 high charge state distribution centering at 49+ (unfolded form) and the second with charges  
204 centered at 30+ (Figure 1E). Both envelopes could be related to different unfolded or partially  
205 unfolded forms. This phenomenon is probably caused by the interaction between proteins in  
206 bicarbonate buffer and CO<sub>2</sub> outgassing during ESI leading to bubbles production. Heating and  
207 bubbling synergistically induce protein unfolding as explained by Hedge and colleagues [22].

208 In our SEC-MS experiments, the dimerization phenomenon of infliximab has been  
209 observed with most of the mobile phases tested except ammonium bicarbonate (Figure 1 B, C,  
210 D). Since ammonium bicarbonate buffer tends to denature proteins, ammonium acetate buffer  
211 (100 mM, pH 6.0) has been chosen.

212

### 213 3.1.2. Influence of organic solvents on FLD and MS signal

214 Organic solvent are often employed in SEC mobile phases to limit the hydrophobic non-  
215 specific interaction of protein to stationary phases, and also to enhance the MS ionization  
216 efficiency [20]. However, a high percentage of solvent could induce; (i) protein denaturation,  
217 (ii) modification of the protein equilibrium between folded and unfolded states, (iii)  
218 fluorescent properties modification and (iv) dissociation of non-covalent oligomers. The  
219 influence of organic solvents on infliximab fluorescent signal has been first evaluated by  
220 adding 10% organic solvent in the compounded infliximab. Both isopropanol (IPA) and  
221 acetonitrile (ACN improved the intrinsic fluorescence intensity of infliximab whereas of the  
222 signal was reduced with methanol (MeOH) (Figure 2A). In addition, a slight shift of emission  
223 maximum (from 345 nm to 337 nm) was observed with MeOH and ACN (Figure 2B). Then,  
224 organic solvents (10 %) were added to the mobile phase (ammonium acetate 100 mM pH  
225 6.0). FLD detection showed that infliximab peak area was higher with ACN compared to IPA  
226 and MeOH (ACN > IPA > MeOH) (Figure 3), while infliximab peak shape and retention time  
227 were similar with the three solvents. With MS detection, IPA addition provided the highest  
228 infliximab signal which was approximately 2 and 5-fold higher compared to MeOH and  
229 ACN, respectively. Therefore, IPA has been chosen for the following experiments. Most  
230 importantly, MS detection showed that a self-association of monomeric mAbs was observed  
231 whatever the organic solvent employed.

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

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

240

### 241 *3.1.3. Influence of mobile phase ionic strength, pH and temperature*

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

### 252 **3.2. Optimization of MS parameters**

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

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

### 270 **3.3. Interaction TNF $\alpha$ and INF**

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

289 suppressing thereby the peak of infliximab monomer. In these latter cases only two different  
290 hetero complexes between TNF $\alpha$  and infliximab were evidenced. As the number of different  
291 detected hetero-oligomers is varying with the increase of the ratios we can hypothesize that  
292 the formation of complexes follows transition states which are TNF $\alpha$ -concentration related.  
293 With the obtained results, we can also conclude that the mobile phase containing 10 % IPA in  
294 150 mM acetate ammonium pH 6.0 do not disrupt the non-covalent interaction between TNF $\alpha$   
295 and infliximab. However, these complexes detected by FLD could not be seen by SEC-  
296 native-MS using ammonium acetate due to the limited mass range detection of QTOF6450 for  
297 the ions above 10 000  $m/z$ . Therefore, in order to confirm the nature of these complexes, the  
298 ammonium bicarbonate buffer inducing protein dissociation of non-covalent complexes  
299 during MS, has been employed to analyze TNF $\alpha$ -infliximab complexes at ratio (1/3) by SEC-  
300 FLD-MS.

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

### 312 **3.4. Analysis of trastuzumab stressed samples**

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

320 In monomer fraction of the stressed sample eluting from 8.8 to 10.3 min (Figure 6B), the  
321 extracted ion current (XIC) showed two peaks ( $t_R$  9.2 and 9.8 min). The extracted-ion  
322 chromatogram (XIC) profile of the monomer eluted at 9.2 min exhibited a main envelop with  
323 a mass/charge ratio ranging from 5000  $m/z$  to 6200  $m/z$  and charge state centered at 27+ that  
324 could correspond to the folded one. This is assessed by the similar charge state displayed by  
325 the fresh trastuzumab sample (centered at 27+) (Figure 6C). The XIC profile of the peak  
326 eluted at 9.8 min (Figure 6B) exhibited a signal corresponding to a degraded form of the  
327 monomer (from 4200 to 5000  $m/z$ ) with a deconvoluted mass corresponding to 104170 Da  
328 (Figure 6D). The slightly higher  $t_R$  of the degraded trastuzumab, could be explained by the  
329 smaller molecular weight of the degraded form. Both species folded and degraded forms  
330 exhibited a poor chromatographic resolution. The dimer exhibited a mass spectrum ranging  
331 from 6000 to 8500  $m/z$  with a deconvoluted mass corresponding to 296 kDa and centered at  
332 41+ (Figure 6E).

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



338 spectrum for the monomer fraction, showed intact antibody having an average molecular  
339 weight of  $148\,455 \pm 40$  Da as well as a degraded form with an average molecular weight of  
340  $101\,207$  Da (Figure 7A). Since ion mobility data were recorded at the same time, we were  
341 able to describe the conformational landscape of the intact antibody (Figure 7B) as well as of  
342 the degraded form (Figure 7C). Figure 7B and 7C display the collisional cross sections (CCS)  
343 of the molecule as a function of the charge state ( $CCS = f(z)$ ), called conformational  
344 landscape. For non-stressed trastuzumab conformational landscape showed that 3 different  
345 conformations (CF1, CF2, CF3) exist in this monomeric fraction. CF3 exhibits a low CCS  
346 ( $3800\text{-}5000 \text{ \AA}^2$ ) and low CSD ranging from 26+ to 28+ which corresponds to folded  
347 monomer. Reversely, CF1 and CF2 exhibit higher CCS (more than  $5000 \text{ \AA}^2$ ) and higher  
348 charges distributions from 28+ to 30+ and 29+ to 31+, respectively for CF2 and CF1  
349 conformers, which correspond to conformers having more expanded structures (Figure 7B).  
350 In contrast, the conformational landscape of the degraded form (Figure 7C) showed that  
351 trastuzumab cleavage lead to the loss of conformers, that can be related to a lower flexibility.

352

#### 353 **4. Conclusion**

354 Here we developed a method based on simultaneous coupling of SEC to ESI-QTOF and (LIF)  
355 FLD suitable for therapeutic mAbs quality control. This method allows us to clearly identify  
356 the experimental conditions leading to artificial conformational changes of mAbs related to  
357 MS and to confirm by native-MS the conformational state of the monomeric peak observed in  
358 FLD (denatured, degraded or folded). Thus, this set-up allows the discrimination between  
359 mAbs dimer artificially formed during the MS analysis from aggregate species originally  
360 present in the sample. This method has been successfully applied to trastuzumab stressed  
361 sample and allowed detecting degraded forms and dimers present in the sample. An online  
362 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

367        **Conflict of interest**

368        The authors declare no competing financial interest.

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370

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377

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471

## Figure captions

472

473 Figure 1: Online SEC-~~FLD~~~~LF~~/native MS analysis of infliximab ( $1 \text{ mg.mL}^{-1}$ ) (A) SEC-  
474 ~~FLD~~~~LF~~ chromatogram using 100mM acetic acid pH 3.0 mobile phase (blue trace) and  
475 100mM ammonium acetate pH 6.0 mobile phase (black trace). Mass spectra of monomeric  
476 infliximab at different mobile phase (B) 100 mM acetic acid pH 3.0, (C) 100 mM ammonium  
477 acetate pH 6.0, (D) 100 mM ammonium formate pH 6.0, (E) 100 mM ammonium bicarbonate  
478 pH 6.0.

479 Column: BioSEC-3, ( $4.6 \text{ mm} \times 300 \text{ mm}$ ,  $300 \text{ \AA}$ , particle diameter  $3 \text{ \mu m}$ ); Flow rate:  $300$   
480  $\mu\text{L.min}^{-1}$ ; ~~FLD~~~~LF~~:  $\lambda_{\text{ex}} = 280 \text{ nm}$ ,  $\lambda_{\text{em}} = 350 \text{ nm}$ . MS condition: drying gas.  $250 \text{ }^\circ\text{C} - 6 \text{ L min}^{-1}$ ,  
481  $^1$ , sheath gas  $200 \text{ }^\circ\text{C} - 6 \text{ L min}^{-1}$ , nebulizer pressure 15 psig, transferred capillary, nozzle,  
482 fragmentor and skimmer voltages were set at 3500 V, 1500 V, 175 V and 65 V respectively

483

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

487

488 Figure 3: Comparison of different organic solvent influence in mobile phase, ACN, MeOH,  
489 IPA. Online SEC-~~FLD~~~~LF~~/native MS analysis of fresh infliximab with mobile phase  
490 containing 10% organic solvent: Mass spectra intensity (orange histogram) and peak area  
491 measured by ~~FLD~~~~LF~~ detector (blue trace) of infliximab monomers.

492 Mobile phase: ammonium acetate 100 mM, pH = 6.0, 10 % organic solvent (v/v), other  
493 experimental conditions as mentioned in Figure 1.

494

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

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

499

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

504 M, D, T, Te are monomeric, dimeric, trimeric and tetrameric forms of TNF $\alpha$ , respectively

505 Mobile phase: ammonium bicarbonate 150 mM pH 6.5 (10% IPA). Column: BioSEC-3, (4.6  
506 mm  $\times$  300 mm, 300 Å, particle diameter 3  $\mu$ m); Flow rate: 300  $\mu$ L.min<sup>-1</sup>; ~~FLDLIF~~:  $\lambda_{ex}$  = 280  
507 nm,  $\lambda_{em}$  = 350 nm.

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

511

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

519 Experimental conditions as mentioned in Figure 5.

520

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 7&:** 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 7A**. **(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.

531