Forced degradation of monoclonal antibodies after compounding: impact on routine hospital quality control.

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Abbreviations: CS = control standard, HMWS= high molecular weight species, PCA= principal component analysis, QC = quality control, UN= unstressed, mab= monoclonal antibody.

Abstract:

Compounded therapeutic monoclonal antibodies (mabs) used in a hospital require quality control (QC). In our hospital, analytical QC process intended to mabs identification and quantification is based on flow injection analysis (FIA) associated with second derivative UV spectroscopy and matching method algorithm. We studied the influence of degraded mabs after compounding on this validated QC. Three forced stress conditions including mechanical, thermal and freeze-thawing stresses were studied to yield degraded mabs from two model compounds, i.e., bevacizumab (IgG1) and nivolumab (IgG4). Different degraded mabs were generated and were analyzed in terms of turbidity, the percentage of aggregation, size distribution, and changes in tertiary structure. Stresses showed to be mab-dependent in terms of aggregation. Tertiary structural changes were observed in most of the stressed samples by principal component analysis (PCA) of the UV second derivative data. The structural and physicochemical modifications conducted to mismatch depending on the nature of the stress. The mismatch ranged from 17 % to 72 % for the mabs, except for freeze-thawed bevacizumab for which a perfect match (100 %) was reached. The quantification with an unfulfilled relative error of the concentration (*i.e.*, $> \pm 15$ %) was detected only for mechanically stressed mabs. In conclusion, the study revealed that the influence of the mabs and the type of stress impact on the QC of compounded mabs.

Keywords: Monoclonal antibody, aggregation, quality control, principal component analysis, freeze-thawing stress, heating stress, mechanical stress, derivative UV spectroscopy.

1 Introduction

2
3 Compounding of anticancer therapies remains a critical activity in the hospital, considering
4 the number of patients to treat due to the increasing prevalence of cancers this past years^{1,2}.
5 Among the therapeutics currently compounded, monoclonal antibodies (mabs) have primary
6 importance in the strategy of anticancer therapy³. Their specificity against targets explains
7 their high interest in this area. Furthermore, their relatively low adverse events enable their
8 use in a wide range of patients. Nowadays, mabs cover various cancer indications such as
9 breast or colorectal cancers, non-Hodgkin's lymphoma, melanoma, and non-small cell lung
10 cancer⁴⁻⁸. The therapeutic use of these drugs requires a compounding stage before patient
11 administration.

12 Mab compounding consists of a dilution of the marketed mabs which are usually available
13 under solution or dried powder with a relatively high concentration (*e.g.*, from 5 to 25
14 mg/mL). According to the good manufacturing practices, each compounded drug should be
15 controlled before the release and patient administration. In the hospital, analytical quality
16 control of these preparations is based on quantification and identification of the mabs before
17 the releasing. However, mabs may face to many stresses^{9,10} along the compounding and
18 storage stages before patient administration. Among them, mechanical or thermal stress may
19 be the most likely encountered in the hospital. Numerous studies have reported the impact of
20 these stresses on mab degradation during various stages such as storage, handling, and
21 administration¹¹⁻¹³. The degradation pathways typically involve chemical and physical
22 degradation^{14,15}. The former may lead to a covalent bond formation, amino acid modification,
23 or oxidation¹⁶. Besides, the latter may allow the formation of aggregates or fragments. Both
24 these degradations alter the integrity of the mab and may limit their bioactivity or increase
25 their immunogenicity¹⁷ threatening their clinical benefices. Indeed, if the clinical
26 consequences of mab aggregates represent a burning issue today and are hardly debated, an

innate and adaptive immune response with highly aggregated mabs, including formulated mabs, has been reported in a population of human peripheral blood mononuclear cells occurring under typical use of manufacturing¹⁸. Consequently, the presence of an important concentration of aggregates becomes a major concern. Besides, structural changes may modify the ability of the mab to interact with its target correctly. Therefore, following and detecting degraded mabs before patient administration is essential.

The analysis of degraded mabs requires orthogonal and complementary techniques in order to characterize them adequately. Size exclusion chromatography (SEC-HPLC) remains the gold standard for the detection of oligomers¹⁹. Other techniques are needed to provide aggregate size information such as, for instance, dynamic light scattering (DLS), flow imaging techniques, or nanoparticle tracking analysis (NTA)^{15,20,21}. Chemical and structural changes are typically analyzed by mass spectrometry, ion exchange chromatography, capillary electrophoresis, gel electrophoresis, spectroscopic techniques (UV, IR, CD, fluoroscopy)²². However, in the context of work in lean production and to face the high demand for outpatient care, fast and high throughput techniques with low handling are necessary. Paul *et al.*, recently reported the interest of automated fluorescence microscopy for high-throughput analysis of submicron aggregates of monoclonal antibodies using a fluorescent dye²³. However, sample pretreatment remains incompatible with the hospital requests since additional steps may delay the QC for the release of the preparation.

Flow injection analysis (FIA) associated with UV spectroscopy using a diode array detector represents an alternative approach. This simple analytical technique is well implemented for quantitation purpose^{24,25}. Also, second derivative spectroscopy allows the study of tertiary structural information through the microenvironment changes of the aromatic amino acid residues in the range 245 – 300 nm^{22,26}. Indeed, phenylalanine, tyrosine, and particularly tryptophan are very sensitive to any perturbation of the microenvironment. Structural change

1 induces a change in magnitude and shifts in the native spectrum²⁷. Such changes in the
2 spectrum can be accurately detected with recent available DAD²². In previous work, we
3 optimized and validated the FIA approach for the identification based on the spectral
4 recognition using simple matching method as well as the quantification of compounded mabs
5 before their infusion²⁸. Identification was performed from the native spectrum of the mabs
6 and their associated match scores. The principle is based on a matching method consisting in
7 the comparison of the spectrum of the analyzed sample and the recorded native spectra from a
8 spectral library leading to a score estimating the degree of similarity between the two spectra.
9 High similarity provides high score typically ranging from 950 to 1000 (perfect match). In the
10 case of a degraded mab, the corresponding spectrum may change due to tertiary structural
11 modifications, and we, therefore, hypothesized that the modified spectral information might
12 lead to mismatch (low match). Indeed, degraded mabs from, e.g., high temperature, freeze-
13 thawing, pH, agitation, or oxidation involve different degradation pathways resulting in
14 different structural modification^{29,30}.

15 This work aimed to analyze the influence of degraded mabs on the analytical QC of the
16 compounded mabs, performed by FIA associated with second derivative UV spectroscopy. To
17 this extent, forced degradations such as mechanical, freeze-thawing and heating stresses were
18 performed in order to obtain different degraded mabs. Mechanical and thermal stress
19 conditions represented the most probable stresses that may be encountered by the mabs before
20 and during the compounding stage at the hospital. Two mabs, bevacizumab (IgG1) and
21 nivolumab (IgG4) which are the most compounded mabs in our hospital were tested for these
22 purposes.

Materials and methods

Materials

Chemicals

1 M sodium hydroxide (NaOH) (VWR international, France), sodium chloride (NaCl) (Sigma-Aldrich, USA), disodium phosphate dihydrate (VWR international, Belgium), sodium dihydrogen phosphate dihydrate (VWR international, Belgium) were of analytical grades. Ultra-pure water was obtained with Mili-Q system from Merck Millipore, France.

Monoclonal antibodies

Two marketed therapeutic monoclonal antibodies, identified from their batch number, were used: bevacizumab (25 mg/mL, Avastin from Roche, Germany) and nivolumab (10 mg/mL, Opdivo from Bristol-Myers Squibb, United-Kingdom).

HPLC instrument

A U-HPLC (UltiMate 3000, ThermoFisher Scientific) equipped with an autosampler, a quaternary pump, and a diode array detector (DAD) was used. The column oven was set at 30°C. The detection was performed from 200 to 400 nm. The data collection and acquisition were carried out with Chromeleon software (V 7.2 SR4, Thermo scientific).

Methods

Monoclonal antibody compounding

Compounding was performed under aseptic conditions using a laminar flow hood. The two mabs were separately diluted at a concentration of 1 mg/mL and a final volume of 50 mL using 0.9% saline FreeFlex®, bags for infusion (PVC (Di(2-Ethylhexyl), phthalate and latex free) from Fresenius Kabi (50 mL, Fresenius Kabi, France). All the compounding bags were

headspace free, stored at 4° C and protected from light for a maximum of 15 days. Sampling from the bags was operated under aseptic conditions with sterile oil free syringes. Samples not subjected to stress conditions are noted as unstressed samples (UN).

Stress conditions

All the experiments were performed using a 4 mL amber glass vial with PTFE cap (Thermo Scientific, USA). Minisart® NML 0.22 µm hydrophilic cellulose acetate filters (Sartorius, Germany) was used when required. For the agitation study, 3.0 mL of the solution of each mab previously prepared were placed in the vial with a PTFE micro-magnetic stir bar (6 x 3 mm). The stress was conducted with an AM 3001 K stirrer (Fisher Bioblock Scientific, France) set at 750 rpm for 4 h. For heat stress, 3.0 mL of the solution of each mab was placed in the vial and heated in a water bath at 50°C for 24 h. Freeze-thawing stress was carried out with 3.0 mL of the solution of each mab placed into the vial and stored in a freezer (-20°C) during 24 h, then heated in a water bath during 5 min at 25°C. The cycle thereby defined were repeated five times in a row. The corresponding samples were denoted as M⁺, T⁺, and FT⁺ for mechanical, heating, and freeze-thawing stresses, respectively. All the stress conditions were performed in triplicates.

Visual inspection

Visual inspection of stressed samples was operated against unstressed samples (standard control) to observe any opalescence and change in clarity under ambient light after direct transfer to clear screw-capped vials.

UV spectrophotometry

A Varian® Cary 50 UV-Vis spectrophotometer (Agilent Technologies, USA) allowed the measurement of UV absorbance of the samples immediately after the stress using a quartz

suprasil[®] cuvette with 10 mm path length (Hellma Analytics, France). All the samples were previously diluted to 0.5 mg/mL with 0.9 % NaCl saline solution when required. Data were recorded considering samples absorbance between 200 and 600 nm with a scanning rate of 600 nm/min. Turbidity was determined from the UV absorbance at 350 nm. The index of aggregation (AI) was estimated following equation²⁹ : $AI (\%) = OD_{350nm} / (OD_{280nm} - OD_{350nm}) \times 100$.

Size-exclusion chromatography (SEC)

The distribution of size variants in the mab samples was analyzed with a TSKgel[®] G3000 SWXL (Tosoh Bioscience, USA) size-exclusion column (7.8 mm ID x 30.0 cm, 5 µm). The mobile phase was 100 mM sodium phosphate buffer containing 200 mM NaCl at pH 6.8. 65 µL of the sample was isocratically eluted for 40 min at a flow rate of 0.4 mL/min. The UV detection was performed at 280 nm. All the samples were previously filtered using a 0.22 µm hydrophilic filter. Peak area integration and data acquisition were achieved with the Chromeleon software. The proportions of the monomer, high and low molecular weight species (HMWS and LMWS) were calculated as the percentage of the total integrated area under the curve (AUC) for the protein peaks. HMWS and LMWS included every peak before and after the monomer peak (main peak) within the excluded volume and the total pore volume. Protein recovery was assessed from the total AUC of the unstressed and nonfiltered mab sample set at 100 %, and the total AUC of the stressed samples.

Flow Injection Analysis

The U-HPLC equipped with stainless steel capillary (0.18 mm internal diameter) was used with a 0.9% saline solution as a carrier stream at a flow rate of 0.5 mL/min. The volume of injection was 2 µL and 10 µL for bevacizumab and nivolumab samples, respectively. FIA was used to both spectral acquisition and quantification purposes of the mab samples as described below.

Second derivative UV spectroscopy and PCA analysis

UV spectra of the mab samples from FIA were collected in the range of 200-400 nm and their corresponding second derivative spectra were calculated using the Savitzky-Golay algorithm from Chromeleon software. The data from the UV second derivative spectra were analyzed by principal component analysis (PCA) to explore trends in data sets according to their tertiary structure.

Quantification of the compounded monoclonal antibodies

The concentration of each compounded mabs before and after the stress was determined by FIA using the validated quantification process as described on our previous study²⁸. Briefly, a calibration curve of the area under the curve of the FIA signal, set at 280 nm, as a function of the concentration ranging from 0.5 to 20 mg/mL and 0.5 to 5 mg/mL was performed for bevacizumab and nivolumab respectively. The validated curves according to the ICH requirements (Q2 (R1)) allowed the calculation of the concentration of the samples. A relative error RE (%) was determined from the expected concentrations.

Identification of the compounded monoclonal antibodies

The identification was carried out using the Chromeleon[®] software from which a least square matching algorithm was performed on the data of the UV second derivative spectra. A matching process was based on the second derivative spectrum of the sample analyzed by FIA and those from a home-made spectral library between 275 nm and 300 nm. A match score ranging from 0 (no similarity) to 1000 (perfect similarity) was calculated. The algorithm assigned the name of the drug for which the spectrum matches with the spectrum of the sample. The approach was optimized and validated in a previous study²⁸.

Statistical analysis

All the stress conditions were tested in triplicates, and appropriate statistical analysis was performed using Mann-Whitney or Wilcoxon tests. Statistical and multivariate analyses were carried out using XLStat data analysis software (Addinsoft, NY, USA).

Results

Unstressed mabs after compounding

The visual inspection, turbidity, and aggregation index (AI) of the fresh solution of bevacizumab and nivolumab (unstressed samples) are summarized in Table 1. These results showed similar characteristics between the two unstressed mab solutions. SEC profile of the fresh unstressed bevacizumab samples (Fig. 1a) showed the presence of monomers and a small fraction of HMWS and LMWS (Table 2). For nivolumab samples, no LMWS has been detected in SEC chromatogram (Fig. 1b) except HMWS and a high proportion of monomers (Table 2). In terms of quantification based on their respective calibration curve, the calculated mean concentration ($n = 6$) of bevacizumab and nivolumab were 1.026 ± 0.003 mg/mL and 0.915 ± 0.003 mg/mL respectively. Considering the expected concentration (1 mg/mL), their calculated relative error (% RE) were 2.5 % and - 9.1 % respectively. Both the compounded mabs fulfilled our laboratory RE requirements set at ± 10 %. For the matching process, the identification of the two mabs was excellent (100 %), and the match scores were higher than 975 (from 978.183 to 992.574 and 984.248 to 992.394 for bevacizumab and nivolumab respectively). Consequently, the two compounded mabs complied with the specifications of our hospital QC.

Physical and chemical characterization of the stressed mabs

Visual inspection, turbidity, and index of aggregation

Visual inspection, turbidity, and index of aggregation of sample solutions of bevacizumab and nivolumab subjected to stresses were assessed. While heat and freeze-thaw did not lead to any change in color or opalescence, both bevacizumab and nivolumab showed an obvious opalescence under stirring (Table 1). The turbidity of the mabs samples was assessed at 350 nm after each stress conditions. For bevacizumab, a significant increase of the turbidity ($p < 0.0001$) was observed in M^+ samples while no difference occurred in FT^+ samples. Surprisingly, a significant decrease in the turbidity ($p < 0.0001$) appeared in T^+ samples. For nivolumab, the turbidity significantly increased ($p < 0.0001$) irrespective of the stress. The % AI was also assessed (Table 1) and M^+ reached the highest level (19.9 % and 20.1 % for bevacizumab and nivolumab respectively). When heated to 50°C, nivolumab showed a significant increase in the AI (+8.7 %) while bevacizumab T^+ samples did not show any significant change as compared to the unstressed samples. FT condition led to the lowest AI (3.4 % and 4.4 % for bevacizumab and nivolumab respectively). According to the results, the AI was mab-dependent for a given stress condition since FT^+ , and T^+ samples from the two mabs showed a significant difference when compared to their respective unstressed solution ($p < 0.0001$).

SEC analysis

SEC analysis was used to determine the size distribution of the species in the samples after the stress conditions. The SEC results describing the percent monomer, HMWS, and LMWS of bevacizumab and nivolumab as a function of the stress conditions are shown in Fig. 1 and Table 2. For bevacizumab, HMWS, monomers, and LMWS were detected irrespective of the conditions (Fig. 1a). All the stressed samples consisted of high monomer content ($> 96,5$ %). Freeze-thaw showed to form HMWS inducing the highest percent HMWS followed by heat

and stirring, which were similar to unstressed samples (Table 2). Fragments that were initially present in the unstressed sample increased very slightly when the mab was heated to 50° C (+ 0.2 %) while FT⁺ and M⁺ showed similar LMWS content as compared to the unstressed samples (Table 2). As depicted in Fig. 2a, the relative recovery was less than 95 % under 50° C (94.1 %) or stirring (91.2 %), indicating the presence of a large amount of aggregates > 50 nm. Freeze-thawing induced a minor fraction of aggregates > 50 nm (1.8 %). For nivolumab, the results of the SEC analysis are shown in Fig. 1b and Table 2. A high monomer content was observed under any stress conditions (> 99 %). Surprisingly, HMWS was almost negligible (~ 0.1 %) after stirring the nivolumab while freeze-thawing and heating did not provide a significant variation of the HMWS content relative to the unstressed nivolumab. LMWS was very low irrespective of the stress conditions. Protein recovery was higher after freeze-thawing (97.7 %) followed by thermally stressed (97.1 %) and stirred (95.9 %) mab (Fig. 2b), indicating the presence of a fraction of large aggregates (> 50 nm) in the stressed samples. Once again, freeze-thaw impacted fewer nivolumab samples.

Second derivative UV spectroscopy

Bevacizumab sample analysis

Second derivative UV spectra of the stressed bevacizumab samples were difficult to visually explore and consequently may lead to subjective interpretation (Fig. 3a). A PCA on the data of the second derivative UV spectra of the unstressed and stressed samples after standard normal variate (SNV) preprocessing was performed to improve the analysis. The PC1-PC3 (17.6%) score plot showed a sharp trend in discrimination between FT⁺ samples and the three other categories of samples along the PC1 explaining 10.4% of the variability (Fig. 3b). Furthermore, the M⁺ samples were negatively correlated to the FT⁺ samples demonstrating distinct microenvironment changes around the three aromatic amino acids of the protein. The most contributing variables on PC1 were from region 275 – 300 nm (275; 285; 288; 292; 295;

297) as shown in Fig. 3c, indicating the high impact of the stress conditions on tyrosine and tryptophan residue regions. Interestingly, the non-stressed samples seem to draw a hyper plan border between the FT⁺ and the M⁺ samples.

Nivolumab sample analysis

The second derivative spectra of these samples provided the expected second derivative UV absorption profile (Fig. 3d). A similar PCA on the data of the second derivative UV spectra of the nivolumab samples including stressed and non-stressed samples was carried out with SNV preprocessing to analyze the structural changes in the range 245 -300 nm. The PC1-PC2 (20.5 %) score plot illustrating the samples according to their stress conditions depicted a less sharp distinction between the stress conditions, particularly the samples from the mechanical stress (Fig. 3e). However, the stressed samples were well separated from the unstressed samples indicating a change in the structure of the degraded nivolumab. The analysis of the contribution of the variables showed the high impact of region 245 – 248 nm on PC1 while most of the variables contributed to PC2 (Fig. 3f).

Identification and quantification of degraded mab samples

Mabs identification

Identification of the mabs was performed by the Chromeleon® software using a matching method after FIA analysis. A partial least square algorithm calculated a match score between the second derivative spectrum of the sample and the spectra of the homemade library. For bevacizumab, the values of the match scores were higher than 975, indicating a good similarity between the two spectra. The lowest match score value was 977.216 corresponding to a match with rituximab. Table 3 detailed the performance of the matching method of the QC before and after filtration of the solution of the stressed mabs. For bevacizumab, the highest mismatch was obtained with M⁺ samples (61.1 %) following by the T⁺ samples (33.3

1 %). Surprisingly, no mismatch occurred with FT⁺ samples where perfect identification (100
2 %) was reached. Filtration improved the matching of the stirred mabs while a slight increase
3 was observed for the heated samples (Table 3). The matching was still perfect for FT⁺
4 samples after filtration. These results indicate that freeze-thawing stress does not interfere
5 with the tertiary structure in the range of 275-300 nm. For nivolumab, the lowest match score
6 value was 983.576 expressing high similarity between the spectra. All the stressed samples
7 resulted in a mismatch where the highest appeared with heat stress (72.2 %) following by
8 freeze-thawing and mechanical stresses respectively (Table 3). The filtration of M⁺ nivolumab
9 unexpectedly increased the mismatch score while it decreased the mismatch for T⁺ and FT⁺
10 samples, as seen in Table 3.

11 *Mab quantification*

12 Quantification of the mabs was carried out by FIA from a validated calibration curve lying the
13 area under the curve of the FIA signal set at 280 nm. The effect of the stress on the
14 quantification measured through the relative errors (RE) from the expected concentration of
15 the mab is shown on Fig. 4. Only mechanical stress yielded to RE > 15 % for both mabs. The
16 other stress conditions yielded to low RE (-5 % ≤ RE ≤ + 5%) lying within the required
17 specifications (*i.e.* - 15 % < RE % < +15 %). The impact of the filtration on the mab
18 quantification was also evaluated. Filtration reduced the RE for each mab, considering the
19 stress conditions (Fig. 4a and b). Such reduction is explained by the presence of aggregates >
20 220 nm. For mechanically stressed bevacizumab, filtration permitted an important reduction
21 of the RE (- 17.5%) while the RE reached -11.3 % and -3.1 % when bevacizumab was filtered
22 after heating at 50° C and freeze-thawing respectively. In the case of nivolumab, a shallow
23 impact of the filtration was observed (RE reaching - 3.7 %, + 0.7 % and - 2.2 % for M⁺, T⁺,
24 and FT⁺ samples respectively). In this experiment, only the filtration of T⁺ nivolumab samples
25 showed a slight increase in the RE. Surprisingly, filtration performed on both unstressed

nivolumab and bevacizumab resulted in a decrease of the concentration (% RE: - 16 % and - 20 % respectively) suggesting an interaction with the filter.

Discussion

As a first step, few stress conditions considered as the most likely exposures were applied in order to provide a set of degraded mabs. Agitation, shake, handling before and after compounding as well as pneumatic tubing system transport were initially considered to comply with mechanical stress (M^+). Inappropriate extended storage in ambient temperature was mimicked by accelerated thermal degradation. Freeze-thawing stress was also experimented to generate another type of degraded mabs. Simple physicochemical assessments were carried out to confirm the production of different populations of degraded mabs. Structural changes were evaluated using second derivative spectroscopy. Change in the native tertiary structure of the degraded mabs has been investigated using PCA through the second derivative UV spectra. A preliminary study with partial to full unfolded bevacizumab or nivolumab obtained from guanidine experiments showed that PCA of the data of the second derivative spectra in the range 245-300 nm, allowed the observation of tertiary structural changes (unpublished data). Moreover, the second derivative spectroscopy considerably reduces interference from scattering³¹. Also, the SNV preprocessing of the data, avoiding any difference in concentration or other influencing effects, allowed focusing on the structural difference between the samples.

Based on the results of the physical characterization, it is clear that different degraded mabs were obtained from the three stresses. Stir stressing of bevacizumab and nivolumab led to higher degraded mabs as compared with freeze-thawing and thermal stressing as seen in Table 1. Although no evaluation of the size of the aggregates was performed, M^+ samples contained a fraction of visible particles ($> 100 \mu m$). It is worth noting that such observation in an IV bag containing mab would not be released. These visible particles demonstrate the

1 harsh conditions of stir stressing. Indeed, a temperature effect due to the friction from the stir
2 bar is added to the mechanical stress increasing the aggregation process. Furthermore, the
3 initial AI of the two mabs (> 3 %) may explain these results. M⁺ samples were therefore
4 considered as the worst case in this present study and were however important to assess since
5 the nature of the aggregates depends on the degradation pathway. Here, this physical stress
6 formed insoluble and soluble aggregates.

7 Freeze-thawing of bevacizumab promoted less degradation as compared to stir
8 stressing with regards to the turbidity (including the resulting AI), and the SEC analysis where
9 the relative protein recovery was much more critical (Fig. 2a). The fraction of aggregates in
10 size range below 50 nm was more significant. Furthermore, the higher percent HMWS in FT⁺
11 samples suggested another aggregation pathway resulting in distinct populations of mabs.
12 Heat stress of bevacizumab generated different types of aggregates where a more substantial
13 fraction of aggregates > 50 nm was formed as seen from the SEC analysis. Additionally, heat
14 showed to induce a fragmentation tendency. Thermally-induced fragmentation was reported
15 by Fincke et *al.* for an IgG1 after a storage temperature of 60° C for 72 H³². Here, the
16 formulated bevacizumab was heat stressed in a lesser extent that may explain a less obvious
17 fragmentation.

18 The PCA performed on the data of bevacizumab in the region 245- 300 nm indicates
19 that structural changes resulting from freeze-thawing were different from stirring. The
20 negative correlation between stirred and freeze-thawed samples proves their high structural
21 discrepancies. Similarly, a spectral difference between thermally stressed and freeze-thawed
22 bevacizumab (Fig. 3b) was observed which denotes a substantial change of the IgG structure
23 between these two populations. The analysis of the PCs showed the importance of the Trp
24 residues which is an excellent probe to inform on structural changes and solvent
25 accessibility^{22,33}. Such results correlate with the study of Hawe et *al.*, suggesting different

aggregation mechanism during freeze-thawing and heating that leads to degraded mabs with distinct physicochemical properties in the case of IgG1¹¹.

For nivolumab, according to AI, visual inspection, and SEC analysis, a significant difference occurred between M⁺, T⁺, and FT⁺ samples. In addition to the presence of large aggregates, SEC analysis revealed a decrease of HMWS in M⁺ nivolumab (Table 2), likely resulting in a distinct degradation pathway (e.g., faster). In comparison to the unstressed and freeze-thawed nivolumab, a different aggregation pathway was suspected due to the high increase of the AI (Table 1). By combining the protein recovery and the AI results, heat stress induced a more considerable amount of aggregates > 50 nm as compared to freeze-thawing stress. The three stresses promoted visible changes in the native tertiary structure of the unstressed sample as revealed the PCA (Fig. 3e). Nevertheless, less distinct groups were reached in the PCA score plot of the spectral data of the stressed samples suggesting minor structural changes between the three types of stressed nivolumab.

Interestingly, when compared to bevacizumab, nivolumab was found to behave differently. Indeed, the higher increase of the AI allows confirming that nivolumab seems to promote larger aggregates when heated. FT stress seems to promote the formation of LMWS on bevacizumab but HMWS on nivolumab as noted through SEC analysis. Besides, stirring led to a decline of HMWS nivolumab changing the initial size distribution (Table 2) while no change in HMWS bevacizumab occurred (Table 2). Furthermore, high-temperature exposure typically generates fragmentation^{34,35}. This tendency was observed only for the IgG1 bevacizumab. Consequently, the degradation is mab-dependent for a given stress. Such differences may be due to, e.g., their IgG subtype, primary structure, or their formulation, demonstrating the complexity of the phenomenons involved in the aggregation process. Finally, we can conclude that different degraded mabs in terms of aggregates quantity, size, and structural modification were obtained from the stress conditions.

1 As the second step, we evaluated the impact of this variety of stressed mabs on the validated
2 QC of the compounded mabs. Both identification and quantitative assessments are required to
3 release a mab preparation. The calculated concentration must be within the limits of the
4 calibration curve which are in agreement with the therapeutic concentrations. Therefore, the
5 LOD/LOQ of our FIA method corresponds to the lowest concentration of the calibration
6 curves.

7 In terms of identification (ID), the value of the match scores calculated from the
8 algorithm (> 975) indicates the good similarity with the retained mab spectrum in the region
9 275 – 300 nm. Except for freeze-thawing of bevacizumab, the ID test shows a potential of the
10 FIA approach combined to the least square matching algorithm to detect degraded mabs. The
11 region 275-300 nm has been validated to explain the highest variability between mabs (e.g.,
12 bevacizumab, rituximab, nivolumab) from our previous study²⁸. Tyrosine and tryptophan
13 residues contribute to the UV absorption in this region. Change in the microenvironment of
14 the buried trp residue leads to shift or magnitude change in the spectrum^{22,26}. Consequently,
15 any structural change in the microenvironment of these amino acid residues within this region
16 leads to a mismatch.

17 Most of the stressed samples mismatched with their expected mab and the degree of
18 mismatch depended on the stress as well as the mab (Table 3). The poor spectral match for
19 M⁺ bevacizumab (filtered or not) indicates that a high degree of structural change can be
20 easily detected. Heated bevacizumab, leading to a fraction of smaller quantity of aggregates in
21 the range 50 -220 nm and a slight difference in the second derivative spectrum was also
22 detected through the mismatch. On contrarily, structural modifications from freeze-thawing
23 observed in PCA, associated with the smallest fraction of aggregates did not affect the
24 recognition of bevacizumab. This fail means a perfect degree of similarity between the FT⁺
25 and unstressed bevacizumab in the region 275 -300 nm. FT seems to be less aggressive and

1 may preserve the humanized IgG1 native structure although the presence of aggregates which
2 may be native-like IgG1 aggregates. These results on ID test are in agreement with Hawe et
3 *al.*, who reported more pronounced structural changes on heated humanized IgG1 than freeze-
4 thawed samples under near and far UV CD, ATR-FTIR spectroscopy and extrinsic fluorescent
5 dyes experiments¹¹. Also, the excipients such as the cryoprotectant trehalose may limit the
6 impact of freeze-thaw.

7 Similarly, stressed nivolumab were also detected as expected from the PCA of the
8 second derivative spectra. However, the ID mismatch was entirely different with nivolumab.
9 This difference demonstrates the influence of the IgG subtype. Indeed, FT, as well as heating
10 stress, considerably affect the ID process (Table 3). Studies on the impact of the IgG subclass
11 on their structural stability reported that IgG4 had lowest thermal stability as compared to
12 IgG1^{36,37}. As a result, subtle structural changes occurring in FT⁺ and T⁺ nivolumab
13 significantly impact the matching. These observations are consistent with the results of
14 Kumru et al., reporting altered tertiary structure under heat with the use of fluorescent ANS
15 dye as a structure change probe³⁸. Curiously, the matching was less impacted when nivolumab
16 was harshly stressed. A possible explanation is that mechanical stress may affect other
17 spectral regions in the case of nivolumab. Indeed, the contribution of the variables 245 to 248
18 was prominent on the PC1 of the PCA on which the mechanically stressed and unstressed
19 samples were highly anticorrelated (Fig 3e and 3f). However, the substantial mismatch
20 difference before and after filtration of the stirred nivolumab (Table 3) shows the complexity
21 of the degradation mechanisms causing the formation of soluble and insoluble aggregates
22 which can be covalent or non-covalent and the resulting structural changes. Further
23 experiments are necessary to better understand this phenomenon. Besides, the detection of
24 freeze-thawed nivolumab after filtration, which is characterized by a high monomer content (
25 > 99 %), with a low quantity of aggregates ranging between 15 – 220 nm, attests the
26 reliability of the FIA ID for detecting degraded IgG4 without noticeable visible particles.

1 More generally, the mismatch is the consequence of the modification of the cation- π
2 interactions between the protein and the sodium chloride carrier stream. Cation- π interactions
3 involve phe, tyr and trp residues³⁹. Unfolding or aggregation of the mabs lead to a substantial
4 change in the solvent accessibility that likely modifies the ability of Na⁺ to diffuse into the
5 interior regions of the mabs. This observation shows the potential interest of the FIA which
6 can be set in single or multiple manifolds (e.g., ions, dyes,...) to perform in-depth
7 investigations of protein structures.

8 Quantitatively, we noted that only harsh stressed mabs were detected by FIA
9 indicating a limit of the quantitative approach if it dissociated from the ID test. QC analysis of
10 the M⁺ samples was unsatisfactory irrespective of the mabs (*i.e.*, RE % > +15 %). The
11 presence of high level of aggregates and particles previously detected undoubtedly causes
12 scattering effects interfering with the UV absorbance measurement. Filtration of the M⁺
13 solution dramatically reduced these effects (Fig. 4) but also the RE (~ -15 %) strengthening
14 the previous results indicating the presence of a fraction of aggregates > 220 nm. For the other
15 stresses, the acceptable RE within the specification (*i.e.* - 15 % < RE % < +15 %) conditions
16 indicates less scattering effect. Moreover, these REs were similar to the RE of the unstressed
17 samples despite the turbidity, particularly for T⁺ nivolumab samples, as seen in Table 1. Even
18 though, aggregation and subsequently, structural change occurred in these samples as
19 described in the first part of the discussion. Then structural modifications induced by these
20 stresses did not significantly impact the availability of the amino acid residues for the UV
21 absorption at 280 nm. The Filtration performed on stressed mabs prior quantitation confirmed
22 the existence of a fraction of aggregates retained by the filter thus decreasing the RE. It is
23 interesting to note that the filtration of FT⁺ bevacizumab has a minor impact on the RE
24 confirming the low quantity of aggregates. Also, taken together, the heated nivolumab AI and
25 filtration data indicate that heat-induced aggregates in size range less than 220 nm.

The filtration of the unstressed samples induced a reduction of the mab concentration (Fig. 4). Furthermore, this decrease was not carried forward to the other stress conditions. Otherwise, the cellulose acetate filter is one of the most recommended filters with low protein binding⁴⁰. Also, the post-filtration AI of the unstressed samples did not significantly increase (data not shown). Consequently, we hypothesized the adsorption of the surfactants, which are more available outside stress conditions, at the solid/liquid interfaces, and the formation of a layer inside the filter impacting the filtration of low volumes of mab solutions. The studies of Mahler et al., and Zhou et al., have reported the adsorption of polysorbates 80 and 20 to a polyethylsulfonate (PES) filter^{40,41}. However, a thorough analysis is needed to provide a better understanding in our case.

In summary, except for mechanically stressed mabs, a QC based only on the quantitative analysis failed to discard stressed mabs and then, need to be conjointly associated with the ID step.

Conclusion

From these experiments, the QC process based on FIA associated with second derivative UV spectroscopy was able to stop structurally altered mabs from mechanical, thermal and freeze-thawing stress. However, this detection depends on the mab subtype as structural modifications from freeze-thawing would not be quantitatively detected as well as qualitatively in the case of bevacizumab. Nevertheless, hospital in-use compounded mab never proceeds freeze-thawing. The QC results according to the stress conditions demonstrate the influence of the complex nature of the monoclonal antibodies and their critical behavior under stress. The selection of an appropriate method for routine QC of the monoclonal antibodies after their compounding is of paramount importance. A QC process based on second derivative UV spectroscopy should be carefully optimized to exclude altered mabs. It is worth noting that further parameters should be taken into account such as, *e.g.*, mabs

concentration and air/liquid surface which impact on the structural change according to the stress justifying the need for extensive studies. Based on this study, the respect of the instructions for mab handling and compounding is of paramount importance to avoid the release of degraded mabs that may be delivered to patients.

The authors declare no conflict of interest.

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1 **Figure captions:**

2 **Figure 1:** SEC profiles of unstressed (UN, blue), freeze-thawed (FT⁺, red), stirred (M⁺,
3 green), and heated (T⁺, purple) bevacizumab (a) and nivolumab (b) at 1 mg/mL.

4 **Figure 2:** percent mass recovery of total protein from the SEC analysis of (a) bevacizumab
5 and (b) nivolumab.

6 **Figure 3:** Second derivative spectra in the range 245 – 300 nm, PCA score-plot and
7 contribution (%) of the variables on the PCs for (a, b, c) bevacizumab (n=60) and (d, e, f)
8 nivolumab (n=59) samples. Unstressed UN (blue diamond), mechanical M⁺ (green triangle),
9 thermal T⁺ (purple cross), and freeze-thawing FT⁺ (red square) stresses. All the data were
10 normalized by SNV preprocessing.

11 **Figure 4:** Quantitative control of the unstressed and stressed bevacizumab (a) and nivolumab
12 (b) before (NF) and after (F) filtration. The relative error is calculated from the calculated and
13 the expected (1 mg/mL) concentration of the mabs.

14

Figure 1

Figure 2

Figure 3:

Figure 4:

Table 1: Visual inspection, turbidity at 350 nm and aggregation index (AI) of bevacizumab (1 mg/mL) and nivolumab (1 mg/mL) samples before (unstressed) and after mechanical (M+), thermal (T+) and freeze-thawing stress conditions.

Sample	Color	Opalescence	Visible particles	Turbidity	AI (%)
<i>Bevacizumab</i>					
Unstressed	colorless	-	-	0.019 ± 0.001	3.3 ± 0.1
M+	white	+++	+++	0.108 ± 0.025	19.9 ± 5
FT+	colorless	-	-	0.019 ± 0.001	3.5 ± 0.2
T+	colorless	-	-	0.017 ± 0.001	3.0 ± 0.2
<i>Nivolumab</i>					
Unstressed	colorless	-	-	0.013 ± 0.01	4.1 ± 0.7
M+	white	+++	+++	0.089 ± 0.032	22.0 ± 6
FT+	colorless	-	-	0.024 ± 0.001	4.4 ± 0.1
T+	colorless	-	-	0.050 ± 0.003	8.7 ± 0.5

Table 2: Average percentage (n =3) of HMWS, monomers, and LMWS for bevacizumab (1 mg/mL) and nivolumab (1 mg/mL) before (unstressed) and after mechanical (M+), heating (T+) and freeze-thawing (FT+) stress conditions determined by SEC analysis.

	% species (mean ± SD)		
	HMWS	Monomer	LMWS
<i>Bevacizumab</i>			
Unstressed	2.0 ± 0.2	97.3 ± 0.2	0.7 ± 0.1
T+	2.0 ± 0.1	97.1 ± 0.1	0.9 ± 0.0
M+	1.9 ± 0.1	97.4 ± 0.1	0.7 ± 0.0
FT+	2.4 ± 0.1	96.8 ± 0.1	0.8 ± 0.1
<i>Nivolumab</i>			
Unstressed	0.5 ± 0.0	99.5 ± 0.0	0.0 ± 0.0
T+	0.5 ± 0.1	99.5 ± 0.1	0.0 ± 0.0
M+	0.1 ± 0.1	99.8 ± 0.0	0.1 ± 0.1
FT+	0.6 ± 0.1	99.3 ± 0.1	0.1 ± 0.0

Table 3: Performance of the identification algorithm of the QC of the compounded mabs before (NF) and after (F) filtration.

samples	Mismatch (%)			
	<i>Bevacizumab</i>		<i>Nivolumab</i>	
	<i>NF</i>	<i>F</i>	<i>NF</i>	<i>F</i>
Unstressed	0	0	0	0
M+	61.1	31.8	16.7	72.2
FT+	0	0	50	44.4
T+	33.3	28.6	72.2	61.1