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Inhaled antibodies: formulations require specific development to overcome instability due to nebulization

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Respiratory infections are life-threatening and therapeutic antibodies (Ab) have a tremendous opportunity to benefit to patients with pneumonia due to multidrug resistance bacteria or emergent virus, before a vaccine is manufactured. In respiratory infections, inhalation of anti-infectious Ab may be more relevant than intravenous (IV) injection – the standard route– to target the site of infection and improve Ab therapeutic index. One major challenge associated to Ab inhalation is to prevent protein instability during the aerosolization process. Ab drug development for IV injection aims to design a high-quality product, stable to different environment stress. In this study, we evaluated the suitability of Ab formulations developed for IV injection to be extended for inhalation delivery. We studied the aerosol characteristics and the aggregation profile of three Ab formulations developed for IV injection after nebulization, with two mesh-nebulizers. Although the formulations for IV injection were compatible with mesh-nebulization and deposition into the respiratory tract, the Ab were more unstable during nebulization than exposition to a vigorous shaking. Overall, our findings indicate that Ab formulations developed for IV delivery may not easily be repurposed for inhalation delivery and point to the requirement of a specific formulation development for inhaled Ab.

Keywords (4-6): monoclonal antibody, inhalation, respiratory tract infections, formulation development, mesh-nebulization

Declarations

- Funding:

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- Conflict of interests/competing interests

NHV is co-founder and scientific expert for Cynbiose Respiratory. In the past 3 years, she received consultancy fees from Argenx, Eli Lilly and research support from Signia Therapeutics, Sanofi and Aerogen Ltd.

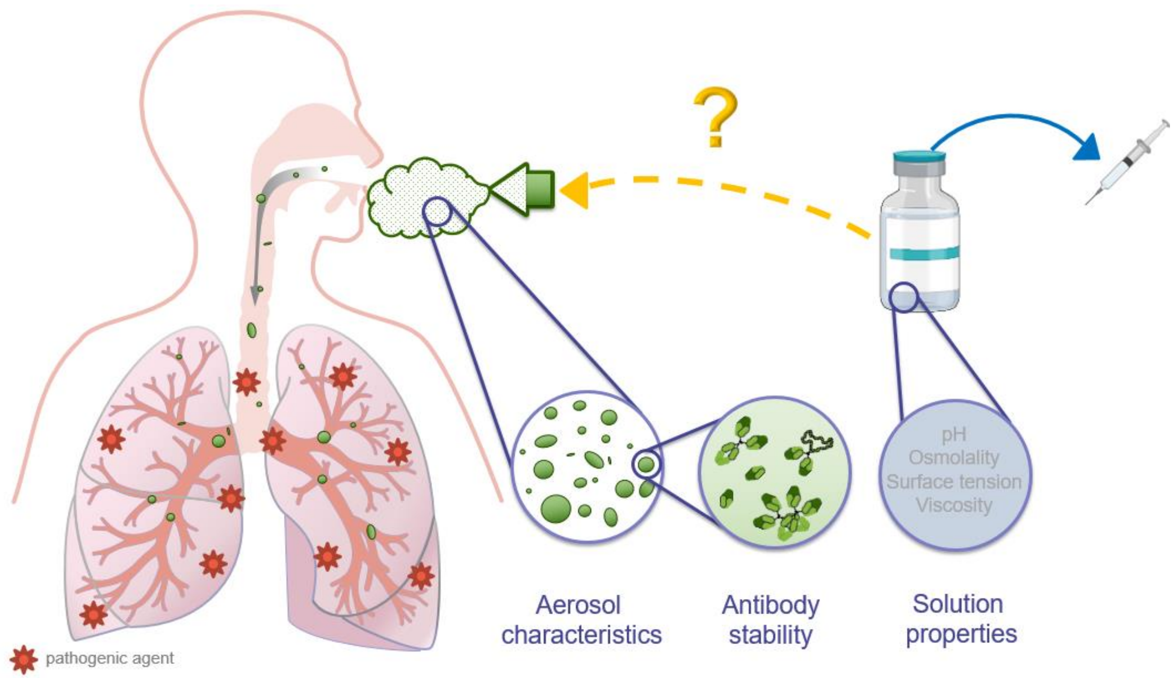
- Ethics approval: not applicable
- Consent to participate: not applicable
- Availability of data and material:

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

- laboratory notebooks filled and signed by the co-workers
- Code availability: not applicable
- Author's contributions:

A.M. participated in the design and implemented all the experiments,
B.T. implemented some experiments
N.HV, R.R, H.A & S.H participated in the design and supervision of research
All authors contributed to the manuscript.

58 **Graphical abstract**



59

60 **Introduction**

61 Acute respiratory infections remain a major health issue, as recently highlighted by the SARS-CoV2 pandemic.
62 Overall, acute respiratory infections are the world's fourth leading cause of death in human of all ages and the first
63 one among children under 5 year-old [1,2]. Although treatments, such as vaccines and antibiotics have been
64 developed, respiratory infections are not under control, mainly because of increasing occurrence of antibiotic-
65 resistant bacteria and emerging viral pathogens. For instance, SARS-CoV2 infection, which can lead to severe
66 pneumonia and respiratory distress syndrome, has already caused more than 2,500,000 deaths around the world,
67 as of March 2021 [3]. Facing this major threat of public health, innovative anti-infective approaches are urgently
68 needed. Among them, therapeutic antibodies (Ab) is a growing class of anti-infective agents, with 3 monoclonal
69 Ab marketed and indicated in respiratory infections, and several molecules targeting respiratory pathogens –
70 including 90 anti-SARS-CoV2 Abs - in development [4–6]. Anti-infective Ab are mostly full-length IgG, acting
71 directly by neutralizing the pathogens and/or stimulating immune responses. As exemplified by the SARS-CoV2
72 pandemic, therapeutic Ab, that do not target the pathogens and are already approved in non-communicable
73 diseases, may also be relevant to prevent respiratory infection-mediated uncontrolled inflammation or abnormal
74 coagulation.

75 Presently, most anti-infective Ab are administered intravenously (IV) [4], but the inhalation route may be more
76 appropriate to improve Ab therapeutic index in respiratory infections, matching the delivery route with the
77 pathogen one, limiting systemic passage and associated risk of systemic toxicity, along with possibly reducing the
78 dose to administer. Inhalation is the mainstay route for drug delivery to treat pulmonary diseases, such as asthma
79 and chronic obstructive pulmonary diseases (COPD) [7,8]. Inhalation consists in delivering a drug as an aerosol -
80 a suspension of 1 to 5 µm-solid particles or -liquid droplets in a gas- directly into the respiratory tract. Most inhaled
81 drugs are small-molecules, like corticosteroids, beta-sympathomimetics, muscarinic antagonists, and antibiotics.
82 The inhalation route remains underexploited for biotherapeutics, with only one protein therapeutics approved so
83 far, Pulmozyme® [9]. Despite inhalation of interferon-β (SNG001, Synairgen) recently achieved positive results
84 in phase 2 clinical trial in hospitalized COVID-19 patients (NCT04385095) [10], the inhalation route is often
85 dismissed/neglected for protein therapeutics because of the lack of supportive clinical data demonstrating its
86 benefit and the challenges associated to inhaled protein development [11–13]. Better understanding the behaviour
87 of inhaled Ab during aerosolization and after deposition into the respiratory tract is critical to support development
88 of appropriate and successful anti-infective Ab products.

89 Pharmaceutical development aims to design a high-quality product and its associated manufacturing process
90 ensuring an efficacious and safe treatment along the life of the product [14]. The formulation scientists play a
91 critical role to produce Ab, with an adequate formulation to ensure shelf life stability and appropriate quality.
92 During formulation development, quality by design principles and ICH guidelines are applied, in particular for
93 biotherapeutics ICH Q1A(R2), Q6B and Q8 (R2) [15–17]. Usually, parenteral (IV or subcutaneous) Ab
94 formulations are developed for storage at 5 °C ± 3 °C for at least two years and should protect the Ab against
95 stress, such as temperature changes, light exposure or shearing associated to shaking during transport and
96 administration to patients. For inhalation, most protein therapeutics in clinical trial are developed as a liquid
97 aerosol, intended to be delivered by nebulization – transforming solutions in micron droplets [9]. During
98 nebulization, proteins are subjected to multiple stress, like temperature rise, exposure to interfaces (liquid-solid or
99 liquid-gas), ultrasound and mechanical shearing [9,18,19]. Ultimately, nebulization can result in Ab aggregation,

100 which may be associated with impaired biological activity and/or unexpected immune responses. As shown in
101 recent published studies, mesh-nebulization is suitable to administer efficiently high dose of proteins into the lungs
102 and limit Ab aggregation [9,18,19].
103 Herein, we hypothesized that liquid formulations developed for intravenous delivery may be appropriate for
104 inhalation delivery, which can facilitate a quick product development to assess the interest of the inhaled route for
105 anti-infectious Ab or repurpose easily relevant IgG molecules during health crisis. In this study, we evaluated the
106 stability of three pharmaceutical Ab formulations during mesh-nebulization in comparison to a vigorous
107 mechanical shaking stress.
108
109 **Material and Methods**
110 ***Antibodies***
111 Three Ab were supplied by Sanofi in their pharmaceutical formulations, compatible for intravenous (IV)
112 administration. They are all full-length IgG1. mAb1 was formulated at 20 mg/mL in histidine buffer at pH 6.0,
113 mAb2 was formulated at 10 mg/mL in histidine buffer at pH 5.8 and mAb3 was formulated at 30 mg/mL in
114 phosphate buffer at pH 6.0. The formulation had the following characteristics: mAb1 2Cp (20°C) and 360
115 mOsm/kg, mAb2 1.3Cp (20°C) and 307 mOsm/kg and mAb3 2.4Cp (20°C) and 306 mOSm/kg. All formulations
116 contained polysorbate 80. Before nebulization, all Ab were filtered to eliminate residual particles (0.22 µm PES
117 syringe filter, Sartorius).
118
119 ***Antibody nebulization and aerosol collection***
120 Two types of vibrating-mesh nebulizers, which are known to limit Ab instability, were used in this study [20]. The
121 Device 1 was a commercial vibrating-mesh nebulizer and the Device 2 was a customized vibrating-mesh nebulizer.
122 Between each nebulization, nebulizers were washed in a bath of hot water (45-55°C) with a detergent product
123 compatible for non-invasive medical devices (Surfanios premium) and rinsed with purified water. Then, 2 mL of
124 purified water were nebulized to remove residual particles and detergent. For Ab nebulization, 2 mL of Ab
125 solutions were introduced in the nebulizer reservoir. The aerosols were collected in a 15-mL sterile conical tube
126 (Corning Life Sciences) by condensation. As recently described, the collection efficiency is approximately 60%
127 [21]. Each Ab was nebulized in triplicate on each device. The nebulized and collected Ab was immediately
128 analysed for aggregation.
129 The performances of the devices were characterized by nebulizing 0.5 mL of NaCl 0.9% or 2.0 mL of Ab
130 formulations and laser diffraction. According to the manufacturer's instructions and our observations, the residual
131 volumes were low (<0.1mL) after nebulization, with any devices and formulations. The concentration of each Ab
132 was determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher), at 280 nm, before and after
133 nebulization to evaluate any change during the nebulization process. During each nebulization, the duration was
134 recorded to determine the flow rate. Each experiment was done in triplicate.
135
136 ***Shaking conditions***
137 Ab solutions (2 mL) were introduced in a 15-mL conical tube (Corning Life Sciences) and agitated with a rotator
138 PTR 60 (Grant bio). The shaking cycle corresponds to successive alternated 360° vertical rotations for 15 seconds

139 at 100 rpm and reciprocal rotations for 45 seconds, for a total of 10 minutes. The rotator was placed in an Ecotron
140 (Infors HT) incubator at 37°C during the entire shaking period. Each experiment was done in triplicate.
141

142 ***Distribution of aerosol droplets by laser diffraction***

143 The volume median diameter (VMD) of aerosol droplets was determined by laser diffraction with a Spraytec
144 (Malvern) equipped with a horizontal inhalation cell (Malvern). The Device 1 was used vertically (normal position)
145 and was connected to the inhalation cell with a T piece. The Device 2 was used horizontally (normal position) and
146 was directly connected to the inhalation cell. The aerosol was aspirated into the inhalation cell with vacuum pump
147 at a flow rate of 15 L/min. The results were expressed as VMD and percentage of droplets with a diameter between
148 1 and 5 µm.
149

150 ***Visual inspection of visible particles***

151 The presence or absence of particles larger than 500 µm was assessed by visual inspection. The samples were
152 placed in a glass vial and illuminated with an MLC-150 cold light source (Motic) on a black background.
153

154 ***Subvisible particles measured by flow-cell microscopy (FCM)***

155 Flow-cell microscopy (FCM) was used to detect particles in the range of 1 µm to 100 µm. All Ab solutions were
156 analyzed before and after nebulization with a particle counting imager Flowcell FC200-IPAC (Occhio) instrument.
157 A volume of 200 µL of each sample was passed through an analysis flow cell and particles were counted and
158 analyzed with the Callisto® software (Occhio). The results were reported as the number of particles per milliter
159 (particles/mL) and expressed as the concentration of all particles (all particles/mL), particles larger than 2µm (>
160 2µm/mL), particles larger than 10µm (> 10 µm/mL) and particles larger than 25µm (> 25µm/mL).
161

162 ***Submicron particles measured by dynamic light scattering (DLS)***

163 Dynamic light scattering (DLS) was used to analyze particles from 50 nm to 2 µm. The measurements were carried
164 out at 25°C, with a DynaPro NanoStar (Wyatt Technology) instrument using a 659 nm laser wavelength and 90°
165 detection angle. Each sample was introduced in a plastic disposable cuvette (Uvette, Eppendorf) and carried out
166 10 acquisitions of 7 seconds. The data were analyzed with Dynamics 7.1.9 software (Wyatt Technology). Samples
167 with more than 30% of the acquisitions rejected were considered non-exploitable, as recommended by the
168 manufacturer. The acceptance criteria were an autocorrelation curve baseline limited at 1 ± 0.01 and a maximum
169 SOS (Sum-Of-Squares error from the correlation function fit) of 100. The results were displayed as the correlogram
170 curves, the monomer radius (nm), percentage of polydispersity of monomer population (%pd), percentage in
171 intensity (% intensity) and in mass (% mass) of the monomer, which resulted from the regularization analysis. The
172 polydispersity index (PDI) resulting from Cumulant analysis and corresponding to the distribution width divided
173 by the mean, was also reported.
174

175 ***Oligomers measured by size-exclusion chromatography (SEC)***

176 Size exclusion chromatography (SEC) was used to analyze oligomers. The measurements were performed on a
177 series 200 (Perkin Elmer) high performance liquid chromatography (HPLC) system. The samples were filtered
178 through a PES 0.22 µm syringe filter into glass vials with reducers. Samples were maintained at 4°C in the storage

179 system before injection. For each sample, 50 µg of Ab was injected by an autosampler on an Advanced BioSEC
180 300 Å, 2.7 µm, 7.8 x 300 mm (Agilent) column. The elution phase was PBS 1X pH 7.2 with 0.03% (w/v) of NaN3
181 perfused at a constant flow rate of 1mL/min at a temperature of 25°C. UV detection was performed at 280 nm with
182 diode array detector. The data were recorded and processed with TotalChrom software (Perkin Elmer). Results
183 were expressed as the percentage of high molecular weights species (% HMW).
184
185 ***Biological activity of mAb1***
186 The measurement of activity is based on the measurement of complement-dependent cytotoxicity (CDC) in the
187 presence of mAb1. mAb1 before and after stress (=samples) and mAb1 reference were diluted at different
188 concentrations and incubated with the antigen carrier cells in the wells of a 96-well plate before adding human
189 complement. Living cells remaining in the wells were visualized with a tretrazolium salt and quantified with a
190 plate reader at 450 nm. The absorbance measured by well is inversely proportional to mAb1 cytotoxicity, which
191 allows to determine a dose-response curve for the samples and the reference. The results are presented as a
192 percentage of CDC activity calculated as a ratio of EC50 value of mAb1 samples and mAb1 reference.
193
194 **Results**
195 **Characterization of aerosols when nebulizing Ab formulations for IV**
196 Pulmonary delivery of Ab can be achieved using several types of devices and, in theory, liquid IV Ab formulations
197 may be directly aerosolized using nebulizers. Herein, we used two vibrating mesh-nebulizers, as this type of
198 devices has been shown to maintain better the stability of IgG during nebulization and can achieve large dose
199 delivery with a high pulmonary deposition [9,18,20]. The performances of the devices with saline solutions and
200 Ab formulations were assessed by measuring the VMD of the aerosols and the flow rates. As reported in Table 1,
201 the flow rates of the two devices with the saline solution were less than 0.5 mL/min and the Device 2 was slightly
202 faster. The flow rates for the two devices decreased in the presence of Ab formulations and the drop was more
203 important with Device 1. The reduction depends on the formulations, with the lowest flow rate observed with
204 mAb3 (30 mg/mL, 2.4Cp (20°C)) essentially with device 1.
205 The VMD of Device 1 and Device 2 with saline solutions were 4.1 µm and 3.5 µm and correspond to a respiratory
206 fraction (particles between 1-5 µm) of 64.4 and 71%, respectively. The VMD was not affected by the nebulization
207 of Ab formulations, except for mAb3 for which the VMD was notably smaller with Device 1, thereby increasing
208 the fine particle fraction (Table 1).
209
210 **Characterization of Ab aggregation when nebulizing pharmaceutical Ab formulations**
211 As previously reported, proteins, including IgG, are highly sensitive to nebulization stress, and the main marker
212 of protein instability during nebulization is aggregation [19,22]. The extent of aggregation depends on the protein
213 nature, the nebulizer and the formulation. Here, we investigated the ability of liquid pharmaceutical formulations
214 compatible for IV administration, which were developed to prevent Ab instability during the product lifespan and
215 to certain environmental stress, to protect Ab from degradation during mesh-nebulization. A vigorous shaking
216 method was used as a control. Aggregation was monitored using orthogonal methods allowing to cover the large
217 range of aggregate populations that can be generated.

218 Not surprisingly, aggregation was dependent on Ab properties and the applied stress. All Ab tolerated the vigorous
219 shaking relatively well. No major generation of visible, subvisible (Figure 1) or submicron (Table 2) aggregates
220 and oligomers (Table 3) has been detected. After nebulization, no visible particle was observed in the samples for
221 the 3 Abs. However, small subvisible particles (<10 µm) were observed by FCM (Figure 1 and Table 2) in samples
222 after nebulization, mostly with Device 2. Although the number of particles was relatively heterogeneous between
223 nebulization runs, device 2 induced a higher amount of aggregates than device 1 (Figure 1 and Table 2).
224 The DLS profiles (Figure 2) displayed slight changes after nebulization for mAb1 and mAb2 as evidenced by the
225 autocorrelation curves. Additionally, except for mAb3 with Device 1, PDI was increased after nebulization (see
226 Table 2) for the 3 Ab, indicating the presence of submicron aggregates. This hypothesis is further supported by
227 the decrease in intensity of the monomer content. However, the lack of decrease of the monomer content in mass
228 suggest that the submicron particles generated during mesh-nebulization represented a very small quantity of
229 particles. SEC results did not highlight important formation of oligomers, except for the nebulized mAb 3, which
230 had a tendency to produce HMW species with Device 2. mAb 1 was one of the most sensitive IgG to nebulization
231 as exemplified by the amount of subvisible particles, %Pd and PDI index observed after nebulization. Because the
232 instability of protein during nebulization may result in some cases in a loss of activity, we analyzed some features
233 of mAb1 activity after nebulization. As shown in Supplemental Table S2, the complement-dependent cytotoxicity
234 of mAb1 was not markedly affected after nebulization.
235
236 **Discussion**
237 Therapeutic Ab, which mainly comprise monoclonal IgG, are one of the most important class of therapeutics and
238 are gaining importance in infectious diseases [4]. Neutralizing the pathogens, inducing anti-infective immune
239 responses and/or preventing excessive inflammation, Ab offer new opportunities for the prevention and treatment
240 of respiratory infections, which still represent unmet medical needs. Several preclinical studies demonstrated the
241 benefit to deliver anti-infective Ab topically to the lungs in viral and bacterial models of respiratory infections
242 [11,23,24] . But the advantage of the inhalation route has not materialized yet in the clinic, thereby emphasizing
243 the challenges to overcome during inhaled Ab development [5]. Among them, the instability of Ab during
244 aerosolization – leading to Ab aggregation- raises both pharmacological and safety issues. The characterization of
245 the best formulation, to ensure the stability of the Ab along its lifespan as a pharmaceutical product, is also a
246 challenge that is faced during intravenous Ab development [25,26]. As a result, formulation scientists select the
247 formulations and explore the Ab stability to various stresses, some of them encountered during aerosolization.
248 Herein, we evaluated the potential use of pharmaceutical formulations, intended for IV delivery, for expanded
249 applications for inhalation.
250 Parenteral protein formulations, including Ab formulations, are being increasingly developed as liquid dosage
251 forms to make them ready to use and to ease use in clinics [14,27]. Thus, numerous IV Ab formulations can be
252 potentially tested in nebulizing systems for inhalation. Previous studies showed that mesh-nebulization was less
253 deleterious on IgG as compared to jet- or ultrasonic-nebulization, most probably because mesh-nebulizers usually
254 display low change in temperature over the nebulization period and no recycling [18,20,28]. However, drug
255 formulations affect mesh-nebulizer performances [28,29]. In particular, ion concentration and increased viscosity
256 were associated with a decrease in droplet size and moderately viscous (>5cP) solutions were not suitable for
257 mesh-nebulization. Herein, the viscosity of Ab formulations was sufficiently low (<5 cP) to enable mesh-

nebulization and overall aerosolization was mostly unaffected by formulation characteristics for the two devices. To a lesser extent, mesh-nebulizer performances may also be influenced by surface tension, which depends on formulation, in particular addition of surfactants [29]. For each device, the proportion of respirable droplets were in the same range, which is in agreement with aerodynamic diameter being similar to the ones obtained with saline solutions. Despite a decrease in flow rate, nebulization times remained within an acceptable time range for administration to spontaneously-breathing patients (<15 min for 2mL solution), with the exception of mAb3 with the device 1. Overall, this means that the 3 IV formulations were compatible with aerosol deposition into the respiratory tract.

Nebulization-mediated aggregation is a serious issue to consider for inhaled Ab. Indeed, aggregation can result in a loss of Ab activity, as the tertiary structure may be impaired, and can elicit, *in vivo*, antidrug-antibody (ADA) production that may neutralize Ab and lead to side effects, such as hypersensitivity responses and anaphylactic reaction. Accordingly, the presence and levels of Ab aggregates along upstream and downstream processes is highly documented for each product. Aggregation covers a broad range of sizes and the European (Eur. Ph. 703) and United States (USP788) pharmacopeias require to evaluate visible particles and subvisible particles over 10 and 25 µm for drug product release [30]. In addition, the regulatory agencies recommend to monitor smaller sized aggregates, including subvisible particles (2-10 µm) and submicronic particles (0.1-2 µm), for full protein product characterization as they may pose a clinical risk [31]. In this study, pharmaceutical Ab formulations subjected to mesh-nebulization resulted in a slight to moderate increase of subvisible particles, mostly those <10 µm. The aggregation profile, as characterized by orthogonal methods, was dependent on the mAb and the mesh-nebulizer. The difference of stability observed with the two mesh-nebulizers may be attributable to heating, shear and mechanical stress inherent to each device or the aerosol size, increasing the air-liquid interface [18,19]. Although IV Ab development takes into account Ab stability to environment stress, such as temperature rise and shearing, pharmaceutical formulations did not seem appropriate for inhalation. Combination of shear stress and local thermal stress appears unique for nebulizers and is likely not enough accurately mimicked by the shaking and thermal stresses as applied for IV formulation development. Our findings are in agreement with those obtained for other parenteral protein formulations [32].

Aggregation may be associated to a loss of activity. Thus, one may question the impact of the aggregates produced during mesh-nebulization on Ab activity. As for mAb1, its biological activity was unaffected by mesh-nebulization (see supplemental Table S1). However, it would be difficult to extrapolate this finding to other Ab, since nebulization-mediated aggregation was inconsistently associated with altered Ab activity [21]. Aggregation is also associated to Ab-related immunogenicity and immunogenicity depends for a part on the route of administration. To the best of our knowledge, the impact of aggregates generated during mesh-nebulization and after inhalation on immune responses has not been investigated yet. But, small-sized subvisible particles (2-10 µm), which correspond to the aggregates mostly produced during mesh-nebulization, have been shown to enhance immune response and are expected to be the most immunogenic [33,34].

It is noteworthy that a vigorous mechanical shaking at elevated temperature was unable to reproduce the combined shear thermal stresses applied in mesh-nebulizer. Overall, the 3 Abs displayed remarkable stability towards strong mechanical shaking. Our results do not match those of Hertel et al. (2014), who defined a shaking method as a surrogate of nebulization stress for protein therapeutics, using one protein nebulized with one device as a study model [32]. In addition to the slight differences between the methods of “shaking at elevated temperature”, the

discrepancy may be attributable to the protein nature and the device. As for us, vigorous shaking at elevated temperature may mimic in some but not all cases, mesh-nebulization. This may be explained by the difficulty to reproduce some nebulization stress by shaking, in particular the huge air-liquid interface generated by pumping the liquid through the mesh and, by definition, in the aerosol droplets. Moreover, local and transient temperature rise in the reservoir of the nebulizer may contribute to additional aggregation. From a formulation scientist's perspective, it means that the vigorous shaking applied in the present study is not suitable to accelerate inhaled Ab development and the study of the stress induced by the device intended for human use has to be performed early in the development process.

Parenteral Ab formulations and particularly intravenous injectable dosage forms require pH and osmolality characteristics basically compatible for inhalation as the lungs tolerate inhaled drug products with osmolality ranging 150–549 mOsm/kg - even if isotonicity has been recommended- and pH ranging 3.5 to 8.0 [35]. Beyond the pH and osmolality, the excipients (nature and dose) in the IV Ab formulations may not be adapted to inhalation and if not used in inhaled drug products they would require toxicity investigations by the inhalation route.

Overall, our findings indicate that Ab formulations developed for IV delivery may not easily be repurposed for inhalation delivery and point to the requirement of a specific formulation development for inhaled Ab. Formulation scientists may select carefully the dose and excipients to be added in the formulation to stabilize Ab during mesh-nebulization, taking into account the paucity of toxicity data on inhaled excipients and their potential impact on formulation properties, and thereby device performances.

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