



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

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Accepted: 22 March 2021 / Published online: 25 March 2021
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Abstract

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 Monoclonal antibody · Inhalation · Respiratory tract infections · Formulation development · Mesh nebulization

Introduction

Acute respiratory infections remain a major health issue, as recently highlighted by the SARS-CoV2 pandemic. Overall, acute respiratory infections are the world's fourth leading cause of death in human of all ages and the first one among children under 5 years old [1, 2]. Although treatments, such as vaccines and antibiotics have been developed, respiratory infections are not under control, mainly because

of increasing occurrence of antibiotic-resistant bacteria and emerging viral pathogens. For instance, SARS-CoV2 infection, which can lead to severe pneumonia and respiratory distress syndrome, has already caused more than 2,500,000 deaths around the world, as of March 2021 [3]. Facing this major threat of public health, innovative anti-infective approaches are urgently needed. Among them, therapeutic antibodies (Ab) are a growing class of anti-infective agents, with 3 monoclonal Ab marketed and indicated in respiratory infections, and several molecules targeting respiratory pathogens—including 90 anti-SARS-CoV2 Abs-in development [4–6]. Anti-infective Ab are mostly full-length IgG, acting directly by neutralizing the pathogens and/or stimulating immune responses. As exemplified by the SARS-CoV2 pandemic, therapeutic Ab, that do not target the pathogens and are already approved in non-communicable diseases, may also be relevant to prevent respiratory infection-mediated uncontrolled inflammation or abnormal coagulation.

Presently, most anti-infective Ab are administered intravenously (IV) [4], but the inhalation route may be more appropriate to improve Ab therapeutic index in respiratory

Héroïse Audat and Nathalie Heuzé-Vourc'h contributed equally to the design and supervision of this work.

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

Pharmaceutical development aims to design a high-quality product and its associated manufacturing process ensuring an efficacious and safe treatment along the life of the product [14]. The formulation scientists play a critical role to produce Ab, with an adequate formulation to ensure shelf life stability and appropriate quality. During formulation development, quality by design principles and ICH guidelines are applied, in particular for biotherapeutics ICH Q1A (R2), Q6B, and Q8 (R2) [15–17]. Usually, parenteral (IV or subcutaneous) Ab formulations are developed for storage at $5\text{ }^{\circ}\text{C} \pm 3\text{ }^{\circ}\text{C}$ for at least 2 years and should protect the Ab against stress, such as temperature changes, light exposure, or shearing associated to shaking during transport and administration to patients. For inhalation, most protein therapeutics in clinical trial are developed as a liquid aerosol, intended to be delivered by nebulization-transforming solutions in micron droplets [9]. During nebulization, proteins are subjected to multiple stress, like temperature rise, exposure to interfaces (liquid-solid or liquid-gas), ultrasound, and mechanical shearing [9, 18, 19]. Ultimately, nebulization can result in Ab aggregation, which may be associated with impaired biological activity and/or unexpected immune responses. As shown in recent published studies, mesh nebulization is suitable to administer efficiently a high dose of proteins into the lungs and limit Ab aggregation [9, 18, 19].

Herein, we hypothesized that liquid formulations developed for intravenous delivery may be appropriate for inhalation delivery, which can facilitate a quick product development to assess the interest of the inhaled route for

anti-infectious Ab or repurpose easily relevant IgG molecules during health crisis. In this study, we evaluated the stability of three pharmaceutical Ab formulations during mesh nebulization in comparison to a vigorous mechanical shaking stress.

Material and methods

Antibodies

Three Ab were supplied by Sanofi in their pharmaceutical formulations, compatible for intravenous (IV) administration. They are all full-length IgG1. mAb1 was formulated at 20 mg/mL in histidine buffer at pH 6.0, mAb2 was formulated at 10 mg/mL in histidine buffer at pH 5.8, and mAb3 was formulated at 30 mg/mL in phosphate buffer at pH 6.0. The formulation had the following characteristics: mAb1 2 Cp (20 $^{\circ}\text{C}$) and 360 mOsm/kg, mAb2 1.3 Cp (20 $^{\circ}\text{C}$) and 307 mOsm/kg, and mAb3 2.4 Cp (20 $^{\circ}\text{C}$) and 306 mOsm/kg. All formulations contained polysorbate 80. Before nebulization, all Ab were filtered to eliminate residual particles (0.22- μm PES syringe filter, Sartorius).

Antibody nebulization and aerosol collection

Two types of vibrating-mesh nebulizers, which are known to limit Ab instability, were used in this study [20]. The device 1 was a commercial vibrating-mesh nebulizer, and the device 2 was a customized vibrating-mesh nebulizer.

Between each nebulization, nebulizers were washed in a bath of hot water (45–55 $^{\circ}\text{C}$) with a detergent product compatible for non-invasive medical devices (Surfanios premium) and rinsed with purified water. Then, 2 mL of purified water were nebulized to remove residual particles and detergent. For Ab nebulization, 2 mL of Ab solutions were introduced in the nebulizer reservoir. The aerosols were collected in a 15-mL sterile conical tube (Corning Life Sciences) by condensation. As recently described, the collection efficiency is approximately 60% [21]. Each Ab was nebulized in triplicate on each device. The nebulized and collected Ab was immediately analyzed for aggregation.

The performances of the devices were characterized by nebulizing 0.5 mL of NaCl 0.9% or 2.0 mL of Ab formulations and laser diffraction. According to the manufacturer's instructions and our observations, the residual volumes were low (<0.1 mL) after nebulization, with any devices and formulations. The concentration of each Ab was determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher), at 280 nm, before and after nebulization to evaluate any change during the nebulization process. During each nebulization, the duration was recorded to determine the flow rate. Each experiment was done in triplicate.

Shaking conditions

Ab solutions (2 mL) were introduced in a 15-mL conical tube (Corning Life Sciences) and agitated with a rotator PTR 60 (Grant Bio). The shaking cycle corresponds to successive alternated 360° vertical rotations for 15 s at 100 rpm and reciprocal rotations for 45 s, for a total of 10 min. The rotator was placed in an Ecotron (Infors HT) incubator at 37 °C during the entire shaking period. Each experiment was done in triplicate.

Distribution of aerosol droplets by laser diffraction

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

Visual inspection of visible particles

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

Subvisible particles measured by FCM

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

Submicron particles measured by DLS

Dynamic light scattering (DLS) was used to analyze particles from 50 nm to 2 µm. The measurements were carried out at 25 °C, with a DynaPro NanoStar (Wyatt Technology) instrument using a 659-nm laser wavelength and 90° detection angle. Each sample was introduced in a plastic

disposable cuvette (Uvette, Eppendorf) and carried out 10 acquisitions of 7 s. The data were analyzed with Dynamics 7.1.9 software (Wyatt Technology). Samples with more than 30% of the acquisitions rejected were considered non-exploitable, as recommended by the manufacturer. The acceptance criteria were an autocorrelation curve baseline limited at 1 ± 0.01 and a maximum SOS (sum-of-square error from the correlation function fit) of 100. The results were displayed as the correlogram curves, the monomer radius (nm), percentage of polydispersity of monomer population (%pd), percentage in intensity (% intensity), and in mass (% mass) of the monomer, which resulted from the regularization analysis. The polydispersity index (PDI) resulting from cumulant analysis and corresponding to the distribution width divided by the mean was also reported.

Oligomers measured by SEC

Size exclusion chromatography (SEC) was used to analyze oligomers. The measurements were performed on a series 200 (Perkin Elmer) high performance liquid chromatography (HPLC) system. The samples were filtered through a PES 0.22-µm syringe filter into glass vials with reducers. Samples were maintained at 4 °C in the storage system before injection. For each sample, 50 µg of Ab was injected by an autosampler on an Advanced BioSEC 300 Å, 2.7 µm, 7.8 × 300 mm (Agilent) column. The elution phase was PBS 1X pH 7.2 with 0.03% (w/v) of NaN₃ perfused at a constant flow rate of 1 mL/min at a temperature of 25 °C. UV detection was performed at 280 nm with diode array detector. The data were recorded and processed with TotalChrom software (Perkin Elmer). Results were expressed as the percentage of high molecular weights species (% HMW).

Biological activity of mAb1

The measurement of activity is based on the measurement of complement-dependent cytotoxicity (CDC) in the presence of mAb1. mAb1 before and after stress (= samples) and mAb1 reference were diluted at different concentrations and incubated with the antigen carrier cells in the wells of a 96-well plate before adding human complement. Living cells remaining in the wells were visualized with a tetrazolium salt and quantified with a plate reader at 450 nm. The absorbance measured by well is inversely proportional to mAb1 cytotoxicity, which allows to determine a dose-response curve for the samples and the reference. The results are presented as a percentage of CDC activity calculated as a ratio of EC₅₀ value of mAb1 samples and mAb1 reference.

Results

Characterization of aerosols when nebulizing Ab formulations for IV

Pulmonary delivery of Ab can be achieved using several types of devices and, in theory, liquid IV Ab formulations may be directly aerosolized using nebulizers. Herein, we used two vibrating-mesh nebulizers, as this type of devices has been shown to maintain better the stability of IgG during nebulization and can achieve large dose delivery with a high pulmonary deposition [9, 18, 20]. The performances of the devices with saline solutions and Ab formulations were assessed by measuring the VMD of the aerosols and the flow rates. As reported in Table 1, the flow rates of the two devices with the saline solution were less than 0.5 mL/min, and the device 2 was slightly faster. The flow rates for the two devices decreased in the presence of Ab formulations and the drop was more important with device 1. The reduction depends on the formulations, with the lowest flow rate observed with mAb3 (30 mg/mL, 2.4 Cp (20 °C)) essentially with device 1.

The VMD of device 1 and device 2 with saline solutions were 4.1 μm and 3.5 μm and correspond to a respiratory fraction (particles between 1 and 5 μm) of 64.4% and 71.0%, respectively. The VMD was not greatly affected by nebulization of Ab formulations for mAb1 and mAb2 for which a slight decrease in VMD value was monitored between device 1 and device 2 as observed with saline. The mAb3 is distinguished by a smaller VMD value with device 1, thus increasing the fine particle fraction compared to device 2 (Table 1).

Characterization of Ab aggregation when nebulizing pharmaceutical Ab formulations

As previously reported, proteins, including IgG, are highly sensitive to nebulization stress, and the main marker of protein instability during nebulization is aggregation [19, 22]. The extent of aggregation depends on the protein nature, the nebulizer, and the formulation. Here, we investigated the ability of liquid pharmaceutical formulations compatible for IV administration, which were developed to prevent Ab instability during the product lifespan and to certain environmental stress, to protect Ab from degradation during mesh nebulization. A vigorous shaking method was used as a control. Aggregation was monitored using orthogonal methods allowing to cover the large range of aggregate populations that can be generated.

Table 1 Results of aerosol characterization with NaCl and Ab in formulations expressed as the mean (n=3) \pm SD of the flow rate, VMD and % (in V volume) of 1 – 5 μm droplets

	NaCl		mAb 1		mAb 2		mAb 3	
	Flow rate (mL/min)	VMD (μm)	Flow rate (mL/min)	VMD (μm)	Flow rate (mL/min)	VMD (μm)	Flow rate (mL/min)	VMD (μm)
Device 1	0.31 \pm 0.02	4.1 \pm 0.1	0.14 \pm 0.02	4.4 \pm 0.2	0.2 \pm 0.03	4.1 \pm 0.2	0.07 \pm 0.01	3.3 \pm 0.3
Device 2	0.47 \pm 0.01	3.5 \pm 0.2	0.37 \pm 0.02	3.4 \pm 0.2	0.27 \pm 0.04	3.0 \pm 0.1	0.29 \pm 0.05	3.9 \pm 0.2
			1 μm < V < 5 μm (%)	1 μm < V < 5 μm (%)	1 μm < V < 5 μm (%)	1 μm < V < 5 μm (%)	1 μm < V < 5 μm (%)	1 μm < V < 5 μm (%)
			64.4 \pm 0.02	4.4 \pm 0.2	56.5 \pm 0.03	4.1 \pm 0.2	57.9 \pm 0.03	70.9 \pm 0.05
			71.0 \pm 0.04	3.4 \pm 0.2	83.8 \pm 0.07	3.0 \pm 0.1	87.6 \pm 0.02	68.1 \pm 0.05

Not surprisingly, aggregation was dependent on Ab properties and the applied stress. All Ab tolerated the vigorous shaking relatively well. No major generation of visible, subvisible (Fig. 1), or submicron (Table 2) aggregates and oligomers (Table 3) has been detected. After nebulization, no visible particle was observed in the samples for the 3 Abs. However, small subvisible particles ($< 10 \mu\text{m}$) were observed by FCM (Fig. 1 and Table 2) in samples after nebulization, mostly with device 2. Although the number of particles was relatively heterogeneous between nebulization runs, device 2 induced a higher amount of aggregates than device 1 (Fig. 1 and Table 2).

The DLS profiles (Fig. 2) displayed slight changes after nebulization for mAb1 and mAb2 as evidenced by the auto-correlation curves. Additionally, except for mAb3 with device 1, PDI was increased after nebulization (see Table 2) for the 3 Ab, indicating the presence of submicron aggregates. This hypothesis is further supported by the decrease in intensity of the monomer content. However, the lack of decrease of the monomer content in mass suggests that the submicron particles generated during mesh nebulization represented a very small quantity of particles. SEC results did not highlight important formation of oligomers, except

for the nebulized mAb 3, which had a tendency to produce HMW species with device 2. mAb 1 was one of the most sensitive IgG to nebulization as exemplified by the amount of subvisible particles, %Pd, and PDI index observed after nebulization. Because the instability of protein during nebulization may result in some cases in a loss of activity, we analyzed some features of mAb1 activity after nebulization. As shown in Supplemental Table S2, the complement-dependent cytotoxicity of mAb1 was not markedly affected after nebulization.

Discussion

Therapeutic Ab, which mainly comprise monoclonal IgG, are one of the most important class of therapeutics and are gaining importance in infectious diseases [4]. In neutralizing the pathogens, inducing anti-infective immune responses, and/or preventing excessive inflammation, Ab offer new opportunities for the prevention and treatment of respiratory infections, which still represent unmet medical needs. Several preclinical studies demonstrated the benefit to deliver anti-infective Ab topically to the lungs in viral and bacterial

A

	mAb 1				mAb 2				mAb 3			
	Before stress	Shaking	Device 1	Device 2	Before stress	Shaking	Device 1	Device 2	Before stress	Shaking	Device 1	Device 2
All particles	5 111 ± 504	2 836 ± 345	15 517 $\pm 13 530$	55 441 $\pm 36 232$	16 380 $\pm 8 908$	29 224 $\pm 20 003$	19 665 ± 793	87 201 $\pm 41 764$	5 705 ± 371	18 595 $\pm 6 765$	13 966 $\pm 5 373$	88 285 $\pm 30 350$
Particles $\geq 2 \mu\text{m}/\text{mL}$	1 002 ± 230	617 ± 200	3 153 $\pm 2 158$	13 508 $\pm 9 023$	3 083 $\pm 1 768$	4 104 $\pm 2 279$	2 209 ± 427	10 012 $\pm 5 902$	1 169 ± 57	2 920 $\pm 1 589$	2 261 ± 855	9 587 ± 4200
Particles $\geq 10 \mu\text{m}/\text{mL}$	31 ± 28	51 ± 11	90 ± 62	1 648 $\pm 1 501$	280 ± 179	244 ± 73	205 ± 160	374 ± 251	58 ± 27	134 ± 69	117 ± 45	349 ± 250
Particles $\geq 25 \mu\text{m}/\text{mL}$	6 ± 11	0 ± 0	13 ± 11	422 ± 547	179 ± 28	73 ± 10	160 ± 0	251 ± 38	18 ± 18	19 ± 19	18 ± 17	67 ± 86

B

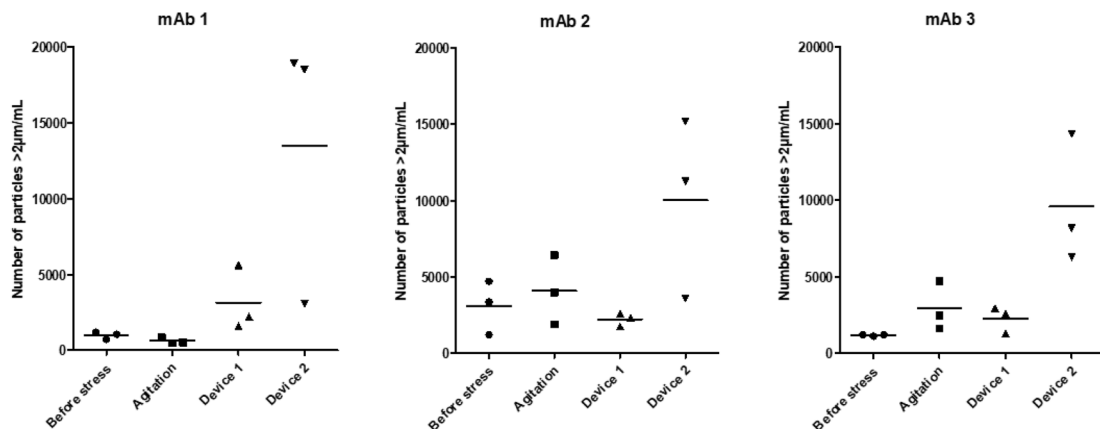


Fig. 1 Subvisible particles measured by flow cell microscopy before and after stress in the mAb formulations. The total number of particles/mL and the number of particles/mL depending on their size are

presented in **a**. All the results correspond to triplicate experiments and are expressed as the mean ($n=3$) \pm SD. Each value of the particles/mL $\geq 2 \mu\text{m}$ and their mean are reported in **b**

Table 2 Results of Dynamic light Scattering, expressed as the mean (n=3) \pm SD of the monomer radius (nm), % pd of the monomer population, % intensity and % mass of monomer and PDI for each Ab formulation before and after stress

	mAb 1			mAb 2			mAb 3					
	Before stress	Shaking	Device 1	Device 2	Before stress	Shaking	Device 1	Device 2	Before stress	Shaking	Device 1	Device 2
radius (nm)	7.5 \pm 0.3	7.0 \pm 0.3	6.7 \pm 0.6	6.9 \pm 0.4	5.5 \pm 0.6	5.0 \pm 0.1	5.0 \pm 0.1	4.9 \pm 0.0	9.3 \pm 0.7	8.7 \pm 0.4	9.2 \pm 1.3	8.8 \pm 0.5
% pd	9 \pm 0.0	16 \pm 0.1	30 \pm 0.1	16 \pm 0.1	9 \pm 0.0	6 \pm 0.0	7 \pm 0.0	9 \pm 0.1	15 \pm 0.0	18 \pm 0.1	11 \pm 0.0	11 \pm 0.0
% monomer (intensity)	100.0 \pm 0.0	99.7 \pm 0.0	95.8 \pm 0.0	96.0 \pm 0.0	100.0 \pm 0.0	100.0 \pm 0.0	99.8 \pm 0.0	98.5 \pm 0.0	100.0 \pm 0.0	100.0 \pm 0.0	100.0 \pm 0.0	99.8 \pm 0.0
% monomer (mass)	100.0 \pm 0.0	99.9 \pm 0.0	99.9 \pm 0.0	99.9 \pm 0.0	100.0 \pm 0.0	100.0 \pm 0.0	100.0 \pm 0.0	100.0 \pm 0.0	100.0 \pm 0.0	100.0 \pm 0.0	100.0 \pm 0.0	100.0 \pm 0.0
PDI	0.113 \pm 0.016	0.102 \pm 0.004	0.214 \pm 0.024	0.219 \pm 0.350	0.071 \pm 0.010	0.070 \pm 0.004	0.114 \pm 0.010	0.157 \pm 0.029	0.119 \pm 0.004	0.134 \pm 0.031	0.132 \pm 0.012	0.162 \pm 0.016

Table 3: Results of size exclusion chromatography, expressed as the mean (n=3) \pm SD of the % of HMW for each Ab formulation before and after stress

	mAb 1			mAb 2			mAb 3					
	Before stress	Shaking	Device 1	Device 2	Before stress	Shaking	Device 1	Device 2	Before stress	Shaking	Device 1	Device 2
% HMW	0.4 \pm 0.0	0.4 \pm 0.0	0.5 \pm 0.0	0.5 \pm 0.0	0.2 \pm 0.0	0.2 \pm 0.0	0.3 \pm 0.1	0.3 \pm 0.0	1.0 \pm 0.0	1.1 \pm 0.1	1.1 \pm 0.0	2.1 \pm 1.7

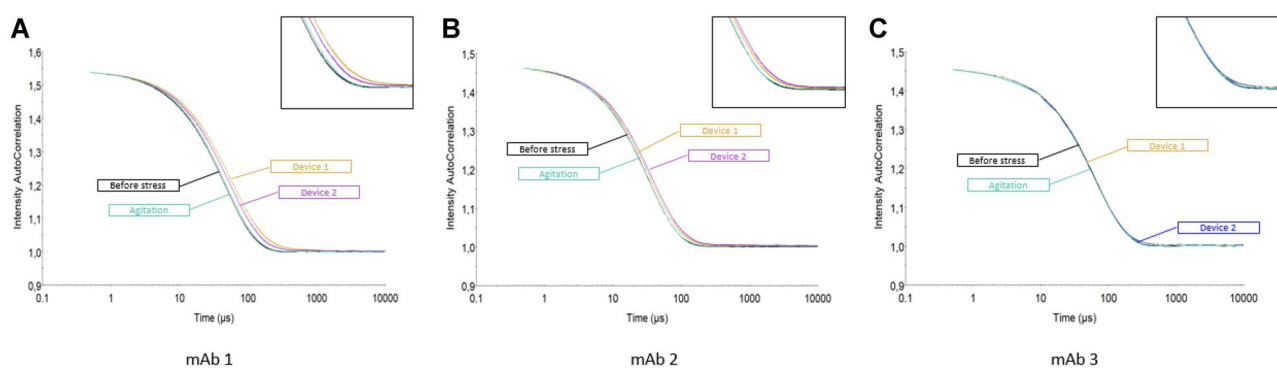


Fig. 2 Correlation curves of Abs in their formulations before and after stress obtained by dynamic light scattering. **a** mAb 1 correlation curves of one representative sample before stress and one after

each stress. **b** mAb 2 correlation curves of one representative sample before stress and one after each stress. **c** mAb 3 correlation curves of one representative sample before stress and one after each stress

models of respiratory infections [11, 23, 24]. But the advantage of the inhalation route has not materialized yet in the clinic, thereby emphasizing the challenges to overcome during inhaled Ab development [5]. Among them, the instability of Ab during aerosolization-leading to Ab aggregation-raises both pharmacological and safety issues. The characterization of the best formulation, to ensure the stability of the Ab along its lifespan as a pharmaceutical product, is also a challenge that is faced during intravenous Ab development [25, 26]. As a result, formulation scientists select the formulations and explore the Ab stability to various stresses; some of them encountered during aerosolization. Herein, we evaluated the potential use of pharmaceutical formulations, intended for IV delivery, for expanded applications for inhalation.

Parenteral protein formulations, including Ab formulations, are being increasingly developed as liquid dosage forms to make them ready to use and to ease use in clinics [14, 27]. Thus, numerous IV Ab formulations can be potentially tested in nebulizing systems for inhalation. Previous studies showed that mesh nebulization was less deleterious on IgG as compared to jet or ultrasonic nebulization, most probably because mesh nebulizers usually display low change in temperature over the nebulization period and no recycling [18, 20, 28]. However, drug formulations affect mesh-nebulizer performances [28, 29]. In particular, ion concentration and increased viscosity were associated with a decrease in droplet size and moderately viscous (>5 Cp) solutions were not suitable for 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 was 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 2 mL 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 are highly documented for each product. Aggregation covers a broad range of sizes, and the European (Eur. Ph. 703) and US (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 a previous study [32], 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. 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.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s13346-021-00967-w>.

Acknowledgements We would like to thank Ronan Crepin (Sanofi) for his technical assistance.

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

Funding This work was supported by the French National Research Agency as part of the “Investissements d’Avenir” program (LabEx MAbImprove, ANR-10-LABX-53-01) and SANOFI. AM was funded by a CIFRE thesis partnership between the CEPR-Inserm U1100 and Sanofi.

Data Availability 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.

Declarations

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

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