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## Protein stability during nebulization: mind the collection step!

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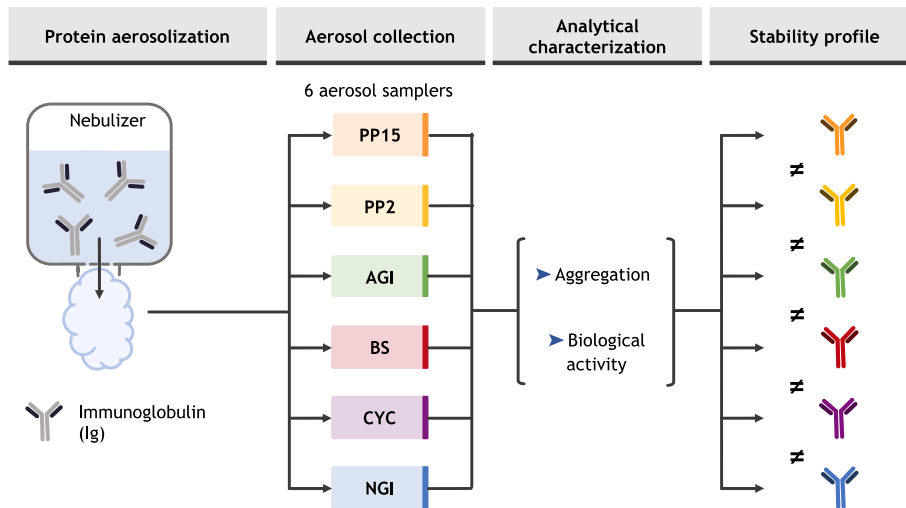
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### Abstract

Inhaled protein therapeutics meet growing interest for the treatment of respiratory diseases. In liquid aerosols, proteins face stresses that may generate instabilities, such as physicochemical denaturations, aggregation and loss of activity. Monitoring protein stability is thus crucial but implies to collect aerosol droplets before analysis. Many aerosol collection methods may be used, still their interference on protein stability is unknown. In this study, we compared the impact of six aerosol samplers on the stability of a model monoclonal antibody (Ig1), aerosolized with a mesh nebulizer. Ig1 stability was assessed for aggregation and biological activity. The six aerosol samplers generated distinct aggregation profiles for Ig1 at all size scales; counts of micron-sized particles varied by a factor of 100. The heterogeneity did not impact Ig1 activity, which was not significantly changed after nebulization. To extrapolate these results, we evaluated the impact of two samplers on three other proteins. Depending on the protein, samplers gave discordant aggregation and/or activity profiles, sometimes in the reverse trend as compared to Ig1. In conclusion, aerosol samplers interfere with protein stability; this impact depends both on the samplers and the protein, highlighting the importance of using the same collection device throughout the aerosol development process.

## Graphical abstract



### Keywords (5-10)

Protein therapeutics; nebulization; aerosols; aerosol collection; stability studies; aggregation.

### Abbreviations (Footnote)

Ig, immunoglobulin; PP15, 15-mL polypropylene tube; PP2, 2-mL polypropylene tube; AGI, all-glass impinger; BS, BioSampler; CYC, Cyclone-BC112; NGI, next-generation impactor; LDH, lactate dehydrogenase.

### Declaration of interest

G. Perret and L. Baptista are employees of LFB Biotechnologies.

## 1. Introduction

Protein therapeutics are considered as promising opportunities in the field of respiratory medicine, bringing significant clinical benefit in several pathologies [1]. While biotherapeutics are most frequently developed for systemic administration, topical lung delivery may offer a number of advantages. Indeed, inhalation has been used for decades for non-invasive pulmonary delivery to improve drug therapeutic index, by reaching optimized targeting of the diseased organ and limit systemic toxicity. This approach seems particularly attractive to take full advantage of protein therapeutics intended to operate within the respiratory tract, for which diffusion from the bloodstream into the lungs is hindered by the pulmonary alveolar capillary membrane [2]. As a matter of fact, several preclinical studies have shown the relevance of inhalation for topical protein delivery, leading to market approval of dornase alpha and ongoing clinical evaluations for about 20 other protein therapeutics [3].

Pulmonary delivery of protein therapeutics can be achieved using several devices such as nebulizers, ~~pressurized metered-dose inhalers~~ soft mist inhalers or dry powder inhalers [3,4]. Among them, nebulizers have been a common choice for first stage of inhaled protein development, as they can achieve large dose delivery, high pulmonary deposition while requiring limited formulation development (by avoiding drying steps, which might be of certain complexity for macromolecules) [5]. Various nebulization studies have been carried out on protein therapeutics, as nicely reviewed by Hertel *et al.* [4]. They underline that nebulization generates stresses when converting bulk liquids into fine inhalable droplets; this includes massive exposition to air-liquid interface, solvent evaporation, heating or aerosol recirculation and recycling [6–8]. These may result in partial to major protein degradation, resulting from physicochemical and conformational changes.

The main marker of protein instability during nebulization is aggregation, which extent depends on the nebulizer and the drug. This type of degradation was reported for various proteins (e.g. lactate dehydrogenase, dornase alpha, G-CSF or monoclonal antibodies), with different nebulization settings (*i.e.* jet, ultrasonic or vibrating-mesh nebulization) [9–14]. Besides, other nebulization-associated degradations have been reported, such as oxidation for IGF-I [15]. For some proteins, such alterations may result in a loss of activity, as was the case for dornase alpha (up to 40% loss with ultrasonic nebulization) or LDH (up to 80% loss with jet nebulization) [11,16]. To limit these instabilities and their potential impact on protein activity and immunogenicity, device and/or formulation optimization have been widely used as stability optimization tools. For instance, dornase alpha was successfully aerosolized with jet and vibrating-mesh nebulizers, allowing full retention of its biological activity [11,17,18]. Likewise,

limiting heating by cooling the nebulizer allowed Hertel *et al.* to improve SM101 stability (a 20 kDa soluble Ig receptor) during vibrating-mesh nebulization [19]. Encouraging results were also obtained with larger proteins: our group showed that careful selection of the nebulizer and formulation optimization (*i.e.* antibody concentration adjustment and addition of surfactants) were key parameters to limit immunoglobulin (Ig) aggregation during nebulization [13].

Thus, the development of nebulized protein therapeutics often requires parallel nebulizer and formulation optimizations. In this context, protein stability studies are the compass that drives the design of the final drug and device combination. Given the complex nature of proteins, their modes of degradation are multiple (changes in primary, secondary and/or tertiary structures) and require the combination of several complementary analytical techniques to achieve extensive characterization. Yet, studying protein stability directly in aerosol droplets is not feasible to date, as standard techniques require volumes in the range of microliters/milliliters. Consequently, aerosol collection is a mandatory step: aerosol droplets are pooled to re-create a bulk liquid for protein characterization.

Numerous devices were developed to collect aerosols, for various applications, mainly bioaerosol sampling and drug aerosol characterization. In such devices, droplet pooling relies on different physical collection mechanisms, such as filtration, liquid/solid impaction, condensation or electrostatic precipitation and have various aerosol collection efficiencies [20]. In the literature, a variety of collection methods has been employed to study protein stability during nebulization, such as glass impingers, laboratory impaction systems, next-generation impactors (NGIs) or filters [12,17,18,21]. Each device implying a specific collection mechanism, proteins may face different post-nebulization stresses depending on the collection process, in addition to general modifications induced by droplet pooling. Together, these factors may modify protein behavior between nebulization and analysis, thereby inducing variations in the results and conclusions of stability studies. Although many studies focused on the consequences of transforming protein solutions into aerosol droplets (during nebulization), data on the impact of re-converting protein aerosols into bulk liquids (during collection) to dissect protein stability are scarce, but critical to interpret nebulized protein stability. To the best of our knowledge, only two comparative studies have been carried out so far: one with dornase alpha, where no difference was observed between two laboratory collection devices [22]. The other one was carried out by Hertel *et al.* on two proteins, with a limited number of devices intended for aerosol collection, and showed different aggregation profiles depending on the collection method [23]. Overall, knowledge about the impact of the aerosol collection system on protein therapeutics behavior remains poor.

Herein, we evaluated the impact of six commercialized aerosol collection systems on the physicochemical and biological stability of a nebulized monoclonal antibody (Ig1). To assess the applicability of our results to other protein therapeutics, we compared two samplers with different profiles on three additional proteins (two Igs and an enzyme). Protein aerosols were produced with mesh nebulizers, which are commonly used for inhaled protein development [24,25]. After aerosol collection, protein stability was assessed in terms of aggregation (from oligomers to visible particles) and biological activity. Protein stability profiles were then established for each aerosol samplers.

## 2. Materials and methods

### 2.1. Materials

HEPES, CaCl<sub>2</sub>, MgCl<sub>2</sub>, bovine serum albumin (BSA), polysorbate 20, polysorbate 80, thimerosal, DNA-methyl green, citric acid, sodium chloride, glycine and phosphate buffered saline (PBS) solution were purchased from Sigma-Aldrich (Saint-Louis, MI). Flow cytometry antibodies were labeled with Alexa Fluor™ 488 (Thermo Fisher Scientific, Waltham, MA). Ig1 isotype-matched nonbinding antibody was purchased from Roche SAS (Boulogne-Billancourt, France). Minimum Essential Media (MEM) without phenol red was purchased from Thermo Fisher Scientific.

### 2.2. Protein solutions

Ig1 (an anti-EGFR IgG1), was supplied by Merck KGaA (Darmstadt, Germany) and diluted to a final concentration of 2 mg/mL in a formulation containing 10 mM citric acid, 100 mM sodium chloride, 0.1% polysorbate 80 and 100 mM glycine. Ig2 (anti-*Pseudomonas aeruginosa* IgG2b) was generated using a PTA-9180 hybridoma (LGC Standards, Molsheim, France) and supplied as a sterile, pyrogen-free solution in PBS, in accordance with good manufacturing practices (BioXCell, West Lebanon, NH); the final concentration was adjusted to 1.8 mg/mL. Ig3 (polyclonal Ig targeting the human Respiratory Syncytial Virus, hRSV) was provided by LFB Biotechnologies (Les Ulis, France) at a concentration of 0.2 mg/mL in a formulation containing mannitol, glycine, and polysorbate 80. Dornase alpha (Pulmozyme™) was purchased from Roche SAS (Boulogne-Billancourt, France) at a concentration of 1 mg/mL in its formulation containing sodium chloride and calcium chloride.

All protein solutions were passed through a 0.22 µm PVDF syringe filter (EMD Millipore, Burlington, MA) and stored at 4°C before nebulization.

## 2.3. Protein nebulization and aerosol collection

### 2.3.1. Generation of protein aerosols

Proteins were aerosolized with mesh nebulizers, which are both favorable to protein stability [24] and suitable with all the evaluated samplers. Briefly, mesh nebulizers produce aerosols by passing a liquid through a calibrated mesh; air supply is thus not needed for aerosol generation. All Igs were nebulized with the Aerogen® vibrating-mesh technology (Aerogen, Galway, Ireland): Aerogen® Solo for Ig1, Ig3 and Aerogen® Pro for Ig2 (this fully-autoclavable nebulizer was selected as Ig2 activity assays required sterile samples). The volume mean diameters (VMDs) were 4.9, 4.1  $\mu\text{m}$  and 4.6  $\mu\text{m}$  for Ig1, Ig2 and Ig3, respectively. For dornase alpha, nebulization was done with an eFlow® rapid (PARI GmbH, Starnberg, Germany), in accordance with the precautions for disposal (VMD = 3.9  $\mu\text{m}$ ) [26].

Except for Ig3, two milliliters of the protein solution were placed in the nebulizer reservoir, and nebulization was carried out. Five replicates were generated for each nebulization/collection condition. For Ig3, the available amount of material allowed us to do 1-milliliter nebulizations, in triplicate.

For each protein, control samples were generated: formulations alone were nebulized and collected following the same process than proteins.

### 2.3.3. Aerosol samplers and collection of protein aerosols

Six aerosol samplers were included in this comparative study, with. They display different characteristics regarding the original application, physical principle of collection, liquid addition and dilution volume, required air flow rate, and existing literature with proteins (see Table 1). Experimental setups are illustrated in Figure 1; in all configurations, nebulizers were connected to aerosol samplers with a T-piece (Aerogen, Galway, Ireland). Aerosols were collected for the entire duration of nebulization, i.e. 4.5 to 9 minutes, depending on the considered protein.

15-mL conical polypropylene (PP) laboratory tubes (Corning Life Sciences, Tewksbury, MA) and 2-mL PP tubes (Dutscher, Brumath, France) were used as aerosol samplers. Empty tubes were connected to the nebulizer outlet through a T-piece in a hermetic setup (the unused T-piece inlet was sealed). Aerosols were collected by condensation on tube walls. No air suction was used and no dilution was applied to samples after collection.

We also used the In tThe AGI-4 Impinger (Ace Glass Inc., Vineland, NJ), a glass device designed for bioaerosol sampling. In this system, droplets are driven through the air flow to

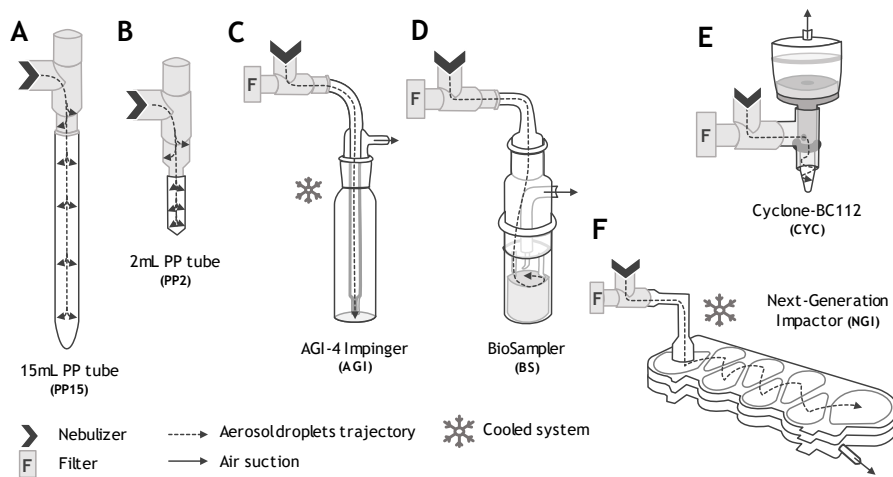
impact at high velocity on the bottom of a glass collection vessel [27]. For our experiments, the AGI-4 was used in a cooled setup [28], plunged in crushed ice; no collection fluid was added in the collection vessel to avoid sample dilution. The AGI-4 was connected to the nebulizer through a T-piece; air was suctioned at 12-12.5 L/min through an Anest-Guard respiratory filter (Teleflex Inc., Wayne, PA) placed on the distal inlet of the T-piece (see Figure 1).

The BioSampler (SKC Inc., Eighty Four, PA), a bioaerosol sampler, was also included in this comparison. This device is similar to the AGI-4, except that aerosol droplets are driven through curved nozzles, placed above a swirling collection fluid. The resulting tangential impaction reduces agitation and re-aerosolization [27,29]. In this study, the BioSampler was used with an air flow rate of 12-12.5 L/min, allowing a -500 mbar depression; incoming air was filtered as for the AGI-4 (see Figure 1). 5 mL PBS were placed in the collection vessel as collection liquid.

The last bioaerosol sampler included in this comparison was the Cyclone-BC112 (NIOSH, Washington, DC), a personal one-stage cyclone sampler, was also included in the study [30–33]. In this device, air flows through a 1.5 mL conical PP tube (Thermo Fisher Scientific) in a vortex-like trajectory, allowing aerosol droplets to impact on the tube walls. Air escapes the sampler after passing through a 3-piece filtration cassette. Air flowrate was set at 4 L/min, suctioned through a respiratory filter placed on the nebulizer T-piece. After nebulization, the collected liquid was recovered by rinsing the tube with 1 mL of protein formulation.

Finally, we included an aerosol was collected in a pharmaceutical aerosol sampler, the NGI (Copley Scientific Limited, Nottingham, UK). It is a seven-stage impactor, in which aerosol droplets are collected after impaction on solid metallic plates. Briefly, each stage contains a series of nozzles of decreasing size. As the air flows through the stages, its velocity increases, leading to selective droplet impaction depending on their aerodynamic size [34]. We used the NGI was used with an air flowrate of 15 L/min [35], filtered by a respiratory filter at the setup inlet. Before aerosol collection, the impactor was pre-cooled for 90 minutes at +4°C [36]. After completing nebulization, each of the 8 plates was rinsed with 5 mL PBS. Samples recovered from the NGI were analyzed both separately and pooled (by mixing equal parts of each element's solution).





**Figure 1. Experimental setups of the evaluated aerosol collection devices.** In each setup, the T-piece was positioned in such a way that aerosol droplet trajectory was the same before reaching the collection device (*i.e.* with a 90° bend).

Name of the sampler (abbreviation)	Original application	Collection characteristics				Literature with proteins*
		Collection mechanism	d <sub>50</sub>	Air flowrate (L/min)	Diluent/liquid addition	
15-mL PP tube (PP15)	N/A	Condensation, sedimentation and impaction	N/D	0	0	
2-mL PP tube (PP2)	N/A	Condensation, sedimentation and impaction	N/D	0	0	[23]
AGI-4 Impinger (AGI)	Bioaerosols	Impaction on solid surface	N/D	12.5	0	[12]
BioSampler (BS)	Bioaerosols	Impaction in liquid	< 0.3 µm [37]	12.5	5 mL	
Cyclone BC-112 (CYC)	Bioaerosols	Impaction on solid surface	1.5 µm [30,31]	4.0	1 mL (added after collection)	
Next-Generation Impactor (NGI)	Therapeutic aerosols (European Pharmacopoeia)	Impaction on solid surface	0.98 µm [38]	15.0	40 mL (added after collection)	[39]

N/D: not determined

\* To the best of the authors' knowledge

**Table 1. Characteristics of the compared aerosol samplers.**

## **2.4. Protein characterization in collected aerosols**

### **2.4.1. Protein concentration and collection efficiency**

Each sampler was weighed before and after aerosol collection, to calculate the volume of aerosol collected. This method being unsuitable with the NGI, the volume of aerosol collected in each element was considered negligible compared to the PBS volume added. Protein concentration in the collected samples was measured using a BCA colorimetric assay (Thermo Fisher Scientific). The total amount of protein collected was obtained by multiplying the final protein concentration by the total volume. Each sampler's collection efficiency was expressed as the ratio: mass of protein collected/mass of protein loaded in the nebulizer.

### **2.4.2. Protein aggregation**

#### *2.4.2.1. Visible particles (visual inspection)*

Samples were observed to assess the presence (+) or absence (-) of visible particles (larger than 500  $\mu\text{m}$ ).

#### *2.4.2.2. Micron-sized particles (flow cell microscopy)*

All protein samples were analyzed before and after nebulization with a FC200S-IPAC protein aggregates counting imager (Occhio SA, Liège, Belgium). Briefly, 240  $\mu\text{L}$  of each sample were passed through an analysis flow cell, where particles between 2 and 100  $\mu\text{m}$  were counted and analyzed in the Callisto® software. Raw results are expressed as the number of particles per mL of solution. When the sampler required to dilute the aerosol (*i.e.* BioSampler, Cyclone and NGI), particle concentration was adjusted with the dilution factor, and thus standardized with respect to protein concentration. Final results are expressed as the number of particles per mL of aerosol, at equal protein concentration.

For each protein, control samples (*i.e.* aerosols of formulation alone) were also analyzed to assess the "particle background" generated by the experimental setup itself. This background was then subtracted from particle counts to get net particle concentrations (*i.e.* protein particles only).

#### 2.4.2.3. Submicronic aggregates (dynamic light scattering)

Dynamic light scattering (DLS) experiments were carried out at 25°C, with a Dynapro Nanostar® instrument (Wyatt Technology, Santa Barbara, CA), as described previously [14]. Briefly, a 659 nm/100 mW laser and a 90° detection angle were used to determine the size distribution profile of protein species before and after nebulization. For each sample, we carried out 10 acquisitions of 7 s each. The results were evaluated using Dynamics® software; samples giving fewer than 70% successful acquisitions were considered to be “not evaluable”, as recommended by the manufacturer. The dynamic data filter was set as follows: baseline limit  $\pm 0.04$  and maximum SOS 1000. For each sample, the global polydispersity index (Pd index corresponds to the distribution width divided by the mean and calculated from the cumulants analysis) was reported, as well as the percentage, in mass, of the monomer, and the percentage polydispersity of the monomer population.

When particle size distribution was multimodal, samples were filtered through a 0.45  $\mu\text{m}$  membrane to remove large particles and re-analyzed.

DLS experiments could not be performed when the dilution factor was too important, *i.e.* for the BioSampler, Cyclone and NGI.

#### 2.4.2.4. Oligomers and soluble aggregates (size-exclusion chromatography)

Size-exclusion chromatography (SEC) analyses were performed on an Ultimate™ 3000 UHPLC system equipped with an in-line. For each sample, 50  $\mu\text{g}$  was injected by an Ultimate™ autosampler in an Agilent Technologies (Santa Clara, CA) Bio SEC-3 column (3  $\mu\text{m}$ , 300 Å, 7.8 x 300mm), and separated at a flow rate of 1 mL/min. The elution buffer was PBS pH 7.2 and UV detection was performed at 280 nm with an Ultimate™ diode array detector (Thermo Fisher Scientific). Results are expressed as the percentages of monomers and higher molecular weight species (HMWS).

SEC experiments could not be carried out when the dilution factor was too important, *i.e.* for Cyclone and NGI.

### 2.4.3. Protein biological activity

#### 2.4.3.1. Ig1 – binding to EGFR

Ig1 binding to its target antigen was assessed through indirect competition in a cell-based assay, as described previously [12]. Briefly,  $1.5 \times 10^5$  A431 cells (human epidermoid carcinoma

cells that express EGFR) were incubated with unlabeled native or nebulized Ig1 ( $1.10^{-6}$  to  $5.10^{-3}$  mg/mL) for 30 min at 4°C. Then, cells were washed twice with PBS + 2% fetal calf serum (FCS) incubated for 30 min at 4°C with FITC-Ig1 (5 µg/mL) or with the isotype-matched nonbinding antibody FITC-Ig (5 µg/mL). After two washes with PBS + 2% FCS, cells were resuspended in 100 µL PBS + 2% FCS + 2 mM EDTA pH8. Flow cytometry analyses were performed using a MACSQuant analyzer (Miltenyi Biotec, Bergisch Gladbach, Germany) and VenturiOne software (Applied Cytometry, Sheffield, UK). Inhibition curves were obtained by plotting mean fluorescence intensity (in % of maximum intensity) against the logarithmic concentration of mAb1; half maximal inhibitory concentration ( $IC_{50}$ ) was calculated for each sample using GraphPad Prism (GraphPad Software Inc., La Jolla, CA).

#### 2.4.3.2. Ig2 – antibacterial activity

Ig2 targets a protein included in *Pseudomonas aeruginosa* type III secretion system, which is used by the bacterium to inject cytotoxic effectors into host cells [40]. Thus, we assessed Ig2 biological activity through a bacterial neutralization assay. Briefly,  $2 \times 10^4$  A549 cells (human adenocarcinomic alveolar basal epithelial cells) were infected with  $2 \times 10^4$  cfu of the PA103 WT-Luc *P. aeruginosa* strain, and native or nebulized Ig2 was added to the medium (100 µg/mL). After 6h incubation at 37°C, supernatants were collected to assay cytotoxicity with a colorimetric LDH release assay (Thermo Fisher Scientific). After 30 min incubation with the reaction mixture, stop solution was added and optical density was read at 490 nm ( $OD_{490}$ ). % cytotoxicity was determined as follows: % cytotoxicity =  $100 \times (\text{Ig2-treated } OD_{490} - \text{spontaneous } OD_{490}) / (\text{positive control } OD_{490} - \text{spontaneous } OD_{490})$ . Spontaneous  $OD_{490}$  was obtained from uninfected cells; positive controls were obtained from uninfected cells incubated with lysis buffer for 45 min. Loss of Ig2 activity was compared before and after nebulization, based on the increase in %cytotoxicity as compared to untreated cells.

#### 2.4.3.3. Ig3 – antiviral activity

Ig3 antiviral activity was assessed by measuring its ability to inhibit hRSV infection of human epithelial cells. To do so, HEp-2 cells (CCL-23) were obtained from ATCC (Manassas, VA, USA) and cultivated in MEM containing 5% FCS and glutamine. Recombinant hRSV was derived from the Long strain and engineered by Marie-Anne Rameix-Welti (INRA UR892, Jouy-en-Josas, France) to express the mCherry red fluorescent protein [41]. Cell infection assays were performed with the resulting hRSV(18)CHERRY 3–P4 virus library (2348 18 541). Insertion of *mCherry* reporter gene between *P* and *M* genes had no major impact on the

replicative capacities of the virus. MEM without phenol red was used for cell culture and virus dilutions.

The day before the test, HEp-2 cells were seeded at  $5 \times 10^4$  cells in 100  $\mu$ L of MEM medium containing 5% FCS. The day of the test, antibody solutions were serially diluted 2.5 fold from 1:2.5 to 1:3814. Equal volumes of 50-fold-diluted virus suspension were added to antibody samples. After 1h incubation at 37°C, 7% CO<sub>2</sub>, virus-antibody mixtures were transferred on cell monolayers. Each sample dilution was run in triplicate, with appropriate positive (LFB03, an anti-RSV-F Ig) and negative (irrelevant Ig) controls. After 36-48h incubation at 37°C in 7% CO<sub>2</sub>, cell infection was assessed by measuring mCherry fluorescence (expressed in relative fluorescence units) on a Tecan infinite M200PRO spectrofluorometer (Tecan, Grödig, Austria) with excitation and emission wavelengths of 580 and 620 nm, respectively. Non-infected HEp-2 cells were used as standards for fluorescence background levels; 100% infection controls consisted in HEp-2 cells incubated with diluted virus suspension only (no antibodies). For each sample, % hRSV infection was plotted against Ig3 logarithmic concentration as a dose-response inhibition of HEp-2 infection; IC<sub>50</sub> was determined using GraphPad Prism.

#### 2.4.3.4. Dornase alpha – enzymatic activity

The enzymatic activity of dornase alpha was assessed using a colorimetric assay that measures the release of methyl green from DNA upon digestion by dornase alpha [18]. Briefly, the DNA-methyl green complex was dissolved in assay diluent (25 mM HEPES, 4 mM CaCl<sub>2</sub>, 4 mM MgCl<sub>2</sub>, 0.1% BSA, 0.05% polysorbate 20 and 0.01% thimerosal, pH 7.5). Standards were prepared by diluting native dornase alpha in assay diluent to concentrations ranging from 10<sup>-5</sup> to 10<sup>-2</sup> mg/mL. Samples were diluted in assay diluent by factors of 1:200, 1:400 and 1:800. Equal volumes of standards, controls and samples were each mixed with the same volume of DNA-methyl green solution and incubated 1h at 37°C under agitation. H<sub>2</sub>O<sub>2</sub> (4 mM) diluted in assay diluent was added and samples were incubated 3h at 25°C under agitation. Finally, absorbances at 620 nm (methyl green absorption maximum) and 492 nm (reference wavelength) were recorded by a SpectraMax® microplate reader and SoftMax Pro software (Molecular Devices, San José, CA). The decrease in A<sub>620nm</sub>-A<sub>492nm</sub> was plotted against the logarithmic concentration of dornase alpha; the average activity from three sample dilutions was obtained as  $\mu$ g/mL and divided by total protein concentration to calculate the %activity.

## 2.5. Statistical analysis

For Ig1, aggregation data (*i.e.* concentrations in micronic aggregates, monomer and HMWS contents and monomer polydispersity) were compared through a 1-way ANOVA, Kruskal-Wallis multiple comparison test. Regarding biological activity, mAb1 inhibition curves were compared before and after nebulization-collection with a 1-way ANOVA, Friedman's test. For the three other proteins, aggregation data and biological activities (*i.e.* % cytotoxicity for Ig2, % hRSV infection for Ig3 and % initial activity for dornase alpha) were compared between PP2 and AGI samples with the Mann-Whitney test. All statistical analyses were done under GraphPad Prism. *P*-values below 0.05 were considered for statistical significance.

## 3. Results

### 3.1. Effects of the aerosol collection method on nebulized mAb1

#### 3.1.1. Aerosol collection performances

For each device, we quantified the proportion of protein loaded in the nebulizer that was recovered in the aerosol sampler after nebulization, named collection efficiency. As exposed in Table 2, all devices had collection efficiencies comprised between 60 and 75%, except CYC which was under 10%. Indeed, after 40-60 seconds of nebulization, this sampler was saturated: droplet impaction stopped, and the aerosol deposited either in the terminal filter of the device, or in the connection between the nebulizer and the CYC.

	Volume of aerosol collected (mL) Mean ± SD	Volume of diluent (mL)	Final protein concentration (mg/mL) Mean ± SD	Collection efficiency % protein collected Mean ± SD
Before nebulization	/	/	2.09 ± 0.17	/
PP15	1.37 ± 0.13	0.0	1.90 ± 0.21	59.9 ± 5.6%
PP2	1.43 ± 0.20	0.0	1.89 ± 0.18	65.5 ± 5.2%
AGI	1.48 ± 0.10	0.0	2.02 ± 0.11	75.1 ± 6.4%
BS	1.46 ± 0.12	5.0	0.52 ± 0.01	73.2 ± 6.1%
CYC	0.16 ± 0.02	1.0	0.31 ± 0.05	8.5 ± 1.5%
NGI	N/D	50.0	0.07 ± 0.01	72.7 ± 5.7%

Table 2. Aerosol collection performances measured for the 6 samplers with 2mL of Ig1 loaded in the nebulizer. [mAb1g1 aerosols had a VMD of 4.9 µm,](#)

Mis en forme : Police :Non Gras

#### 3.1.2. Effect of the aerosol samplers on Ig1 aggregation

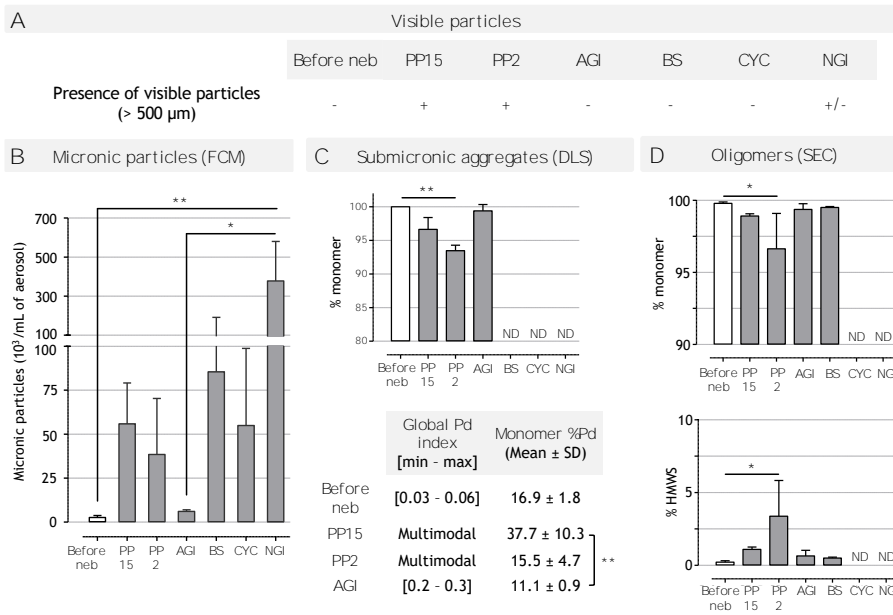
Aggregation is one of the main markers of protein instability. It results from the clustering of several native or denatured protein molecules, generating structures ranging from soluble dimers (several nanometers) to micron-sized insoluble particles [42,43]. Thus, protein aggregation is commonly assessed by a combination of complementary analytical techniques, with different principles of analysis and particle size ranges [44,45]. Herein, we used visual inspection for particles above 500  $\mu\text{m}$ , flow cell microscopy for micron-sized particles, DLS for submicronic aggregates and SEC for protein monomer and oligomers.

Aggregation results were heterogeneous among the tested aerosol samplers. Visible particles were constantly observed in PP15 and PP2 samples, but not for AGI, BS or CYC (see Figure 2A). For the NGI, this particle population was observed only on certain impaction plates, namely the ones that collect droplets < 1.36  $\mu\text{m}$ , despite lower amounts of deposited protein (see Figure S1). However, these results were not predictive of smaller-scale aggregation, as evidenced by concentrations in micron-sized aggregates (Figure 2B). Indeed, PP15 and PP2 concentrations were within the same order of magnitude than BS and CYC (which presented no visible particles), roughly 10 times higher than AGI and 10 times lower than NGI samples. Overall, the range of concentration in micronic aggregates varied amongst aerosol samplers by a factor of 100, with a significant difference between AGI and NGI. In the submicronic range (Figure 2C), all DLS profiles were modified after nebulization, as evidenced by global Pd indexes. Again, the aerosol samplers led to different results: while AGI samples had increased Pd indexes, low polydispersities and a monomer content similar to the initial solution (99.4 vs 100.0%), aerosol collection in PP2 led to a significant decrease in monomer proportion (-6.5%). Besides, the global Pd index was not measurable in this condition, indicating a multimodal distribution of submicronic protein particles. Interestingly, PP15 samples presented a singular profile: even though the decrease in monomer content was limited (-3.4%), the polydispersity was significantly higher than for AGI samples (37.7 vs 11.1%, respectively), suggesting the presence of dimers or oligomers. Finally, SEC results were concordant, indicating a higher loss in monomer for PP2 than PP15 and AGI samples (-3.2%, -0.9% and -0.4%, respectively), originating from a significant enrichment in higher molecular weight species. DLS and SEC data could not be obtained when final protein concentrations were too low (BS, CYC and NGI for DLS, CYC and NGI for SEC).

Overall, Ig1 aggregation was profile modified after nebulization, each sampler affecting specific particle/aggregate populations. While AGI collection seemed to raise essentially micron-sized particles, PP2 induced marked protein aggregation, associating significant monomer loss, micron-sized and visible particles. Surprisingly, PP15 and PP2 profiles were very close for micronic particles, but not for monomers and oligomers; BS, CYC and NGI characterization, although incomplete, showed different behaviors regarding particles over 1  $\mu\text{m}$ . Overall, as



protein aggregation cannot be assessed in the aerosol, it is difficult to conclude on the specific bias generated by each aerosol sampler.



**Figure 2. Effects of aerosol samplers on Ig1 aggregation.**

**A.** Presence/absence of visible particles in Ig1 before/after nebulization. (-): absence / (+): presence / (+/-): inconstant presence (depending on the NGI plate). **B.** Concentrations in aggregates  $\geq 1\mu\text{m}$  obtained by flow cell microscopy. For each condition, the number of particles in blank aerosols (*i.e.* formulation alone) was subtracted. **C.** SEC determination of the content in protein monomer, expressed as % of all detected species. Due to low final protein concentrations, CYC and NGI samples could not be analyzed. **D.** DLS determination of the content in protein monomer, expressed as % of all detected species. Due to low final protein concentrations, BS, CYC and NGI samples could not be analyzed.

Results are presented expressed as means  $\pm$  one standard deviation SD between of the five replicates.

Before neb: antibody solution before nebulization; Pd: polydispersity; HMWS: higher molecular weight species; ND: no data available.

### 3.1.3. Effect of the aerosol samplers on Ig1 biological activity

To assess Ig1 biological activity, we compared its affinity for its target (EGFR), pre- and post-nebulization. To do so, we carried out an indirect competition assay on EGFR-expressing cells,

to evaluate the ability of Ig1 to displace the interaction between EGFR and fluorescent-labeled Ig1. Incubation of different Ig1 concentrations allowed us to build dose-inhibition curves for FITC-Ig1 binding and calculate the IC<sub>50</sub> of each sample. As shown in Table 3, IC<sub>50</sub> were very similar before and after nebulization, regardless of the collection method. Thus, nebulization and collection did not seem to have any impact on Ig1 biological activity.

	<b>FITC-Ig1 binding IC<sub>50</sub> (mg/mL)</b>
<b>Before nebulization</b>	2.2 10 <sup>-4</sup>
<b>PP15</b>	1.8 10 <sup>-4</sup>
<b>PP2</b>	2.0 10 <sup>-4</sup>
<b>AGI</b>	1.7 10 <sup>-4</sup>
<b>BS</b>	1.8 10 <sup>-4</sup>
<b>CYC</b>	1.9 10 <sup>-4</sup>
<b>NGI</b>	2.1 10 <sup>-4</sup>

**Table 3. Effect of aerosol samplers on Ig1 affinity for its target after nebulization.**

### **3.2. Applicability to other protein therapeutics**

In a second part, we investigated whether the observed trends may be generalized to other protein therapeutics. To do so, we selected two of the six aerosol samplers and tested their impact on the physicochemical and biological stability of three nebulized proteins. Selection criteria included: distinct mechanism of aerosol collection, low/no sample dilution (to be suitable with all analyses) and distinct profiles regarding Ig1 stability. Based on these criteria, we selected PP2 and AGI for this part, because they did not dilute samples and gave different aggregation patterns for Ig1.

#### **3.2.1. Aerosol collection performances**

As previously done with Ig1, we compared the aerosol samplers in terms of aerosol collection performance (see Table 4). Depending on the considered protein, collection efficiencies were variable regarding protein concentration, volume and final collection efficiency. Overall, AGI samples contained higher protein concentrations (76-112% of the initial solution concentration) than PP2 samples (54 to 94% of the initial solution concentration). Compared with Ig1, where PP2 and AGI concentrations were respectively 90% and 97% of the initial concentration, greater variations were observed with these 3 proteins, resulting in some cases in substantial protein up-concentration or dilution. Regarding volumes, the two samplers behaved in a similar

way, except with Ig2 which was associated with lower collected volumes for the AGI (56%) than the PP2 (72%). Resulting collection efficiencies were variable and heterogeneous between the three proteins, with slightly higher efficiencies for the AGI than PP2. Globally, PP2 and AGI gave different results in terms of collection characteristics, depending on the considered protein.

Aerosol sampler	Protein therapeutic	Final protein concentration (% initial) Mean $\pm$ SD	Volume collected (% loaded) Mean $\pm$ SD	Collection efficiency % protein collected Mean $\pm$ SD
PP2	Ig1	90.4 $\pm$ 8.6	71.5 $\pm$ 10.0	65.5 $\pm$ 5.2
	Ig2	54.4 $\pm$ 24.7	72.0 $\pm$ 3.7	39.4 $\pm$ 18.6
	Ig3	79.1 $\pm$ 5.1	82.7 $\pm$ 4.0	65.5 $\pm$ 7.2
	Dornase alpha	93.8 $\pm$ 5.4	77.5 $\pm$ 7.7	72.8 $\pm$ 9.4
AGI	Ig1	96.7 $\pm$ 5.3	74.0 $\pm$ 5.0	75.1 $\pm$ 6.4
	Ig2	97.1 $\pm$ 12.7	56.0 $\pm$ 7.2	53.7 $\pm$ 1.2
	Ig3	75.7 $\pm$ 8.3	90.0 $\pm$ 8.7	67.9 $\pm$ 6.6
	Dornase alpha	112.1 $\pm$ 4.3	72.2 $\pm$ 7.1	80.8 $\pm$ 5.9

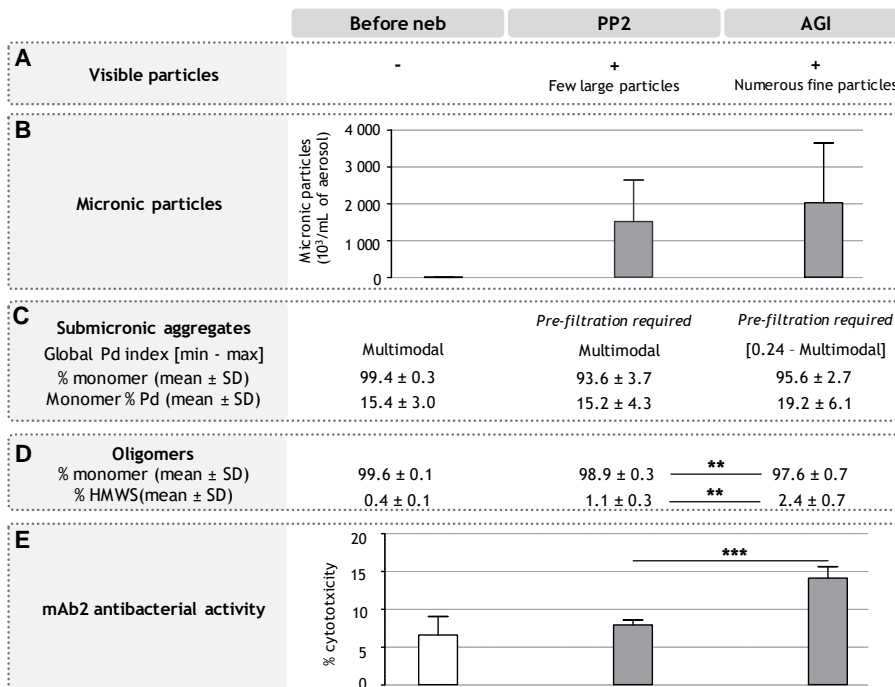
**Table 4. Aerosol collection performances depending on protein therapeutics.** Aerosol collection performances were measured for PP2 and AGI with Ig2, Ig3 and dornase alpha; data obtained with Ig1 are recalled for each device. Aerosol characteristics were similar for all proteins, with VMDs of [3.9 - 4.9]  $\mu$ m and nebulization output rates of [0.31 – 0.50] mL/min.

### 3.2.2. Comparison of PP2 and AGI on nebulized Ig2 stability

Ig2 aerosols were produced from a 1.8 mg/mL solution, nebulized with an Aerogen® Pro mesh nebulizer. As for Ig1, nebulized Ig2 physicochemical stability was monitored through aggregation, by combining 4 orthogonal analytical techniques. In the range of large aggregates, both PP2 and AGI produced visible particles, but with distinct profiles: PP2 produced larger particles than AGI, but in fewer quantity (see Figure 3A). This difference was not retrieved for micronic aggregates, with very similar concentrations for the two aerosol samplers (see Figure 3B). Accordingly, at the submicron scale, PP2 and AGI displayed similar behaviors. In both cases, 0.45  $\mu$ m pre-filtration was required for DLS analysis, and the global Pd index was not measurable, suggesting a multimodal profile of particle populations. Besides, monomer loss (-4-6%) and polydispersity (15-19%) were comparable (see Figure 3C). In contrast, AGI samples contained significantly higher proportions of HMWS, as indicated by

SEC data (see Figure 3D). These results seem to indicate that, in spite of close profiles at the micrometer scale, PP2 and AGI produced distinct populations of aggregates in the ranges of visible particles and oligomers.

Finally, nebulized Ig2 activity was quantified through a bacterial neutralization assay. Herein, we assessed the ability of Ig2 to reduce *P. aeruginosa*-induced cytotoxicity on lung epithelial cells after 6h incubation. As shown in Figure 3E, Ig2 aerosols collected with AGI were associated with significantly higher levels of cytotoxicity than those collected with PP2 (14.1% vs 8.0%, respectively), indicating a lower biological activity. Interestingly, Ig2 biological activity was unchanged by nebulization and collection with PP2.



Pd: polydispersity; HMWS: higher molecular weight species

**Figure 3. Ig2 aggregation and antibacterial activity after nebulization and collection with PP2 and AGI.** Results are presented expressed as means ± one standard deviation between SD of the five replicates.

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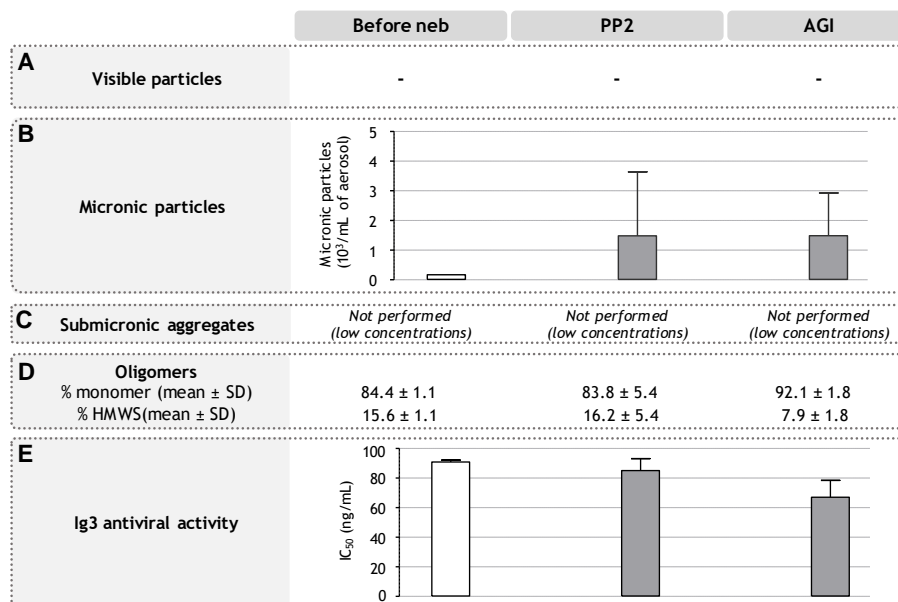
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### **3.2.3. Comparison of PP2 and AGI on nebulized Ig3 stability**

Ig3, a polyclonal antiviral Ig, was nebulized at a concentration of 0.2 mg/mL using an Aerogen® Solo nebulizer. Ig3 aggregation profile was very comparable between PP2 and AGI at all size scales, with no visible particle and low concentrations in micronic aggregates (see Figures 4A and 4B). Even though DLS could not be performed due to low protein concentration, SEC data also suggest similar profiles in terms of monomer and HMWS contents (see Figures 4C and 4D). Interestingly, AGI samples contained slightly higher proportions in monomer (and lower amounts of HMWS) than the non-nebulized antibody solution, but these differences were not significant.

Finally, Ig3 antiviral activity was measured as its ability to neutralize hRSV and thus reduce HEP-2 cell infection *in vitro*. Dose-response inhibition curves were obtained from incubation with various Ig3 concentrations and led to IC<sub>50</sub> determination for each condition. As shown in Figure 4E, AGI-collected samples had a slight (but not significant) difference in IC<sub>50</sub> value as compared to non-nebulized and PP2 samples. Besides, higher variabilities in inhibition curves were observed for AGI samples (data not shown).

Combined with aggregation profiles, these results indicate that nebulized Ig3 behaves similarly when collected with AGI or PP2. However, we may not rule out differences since the low initial protein concentration resulted in incomplete sample characterization (DLS could not be performed) and low particle counts.



Pd: polydispersity; HMWS: higher molecular weight species

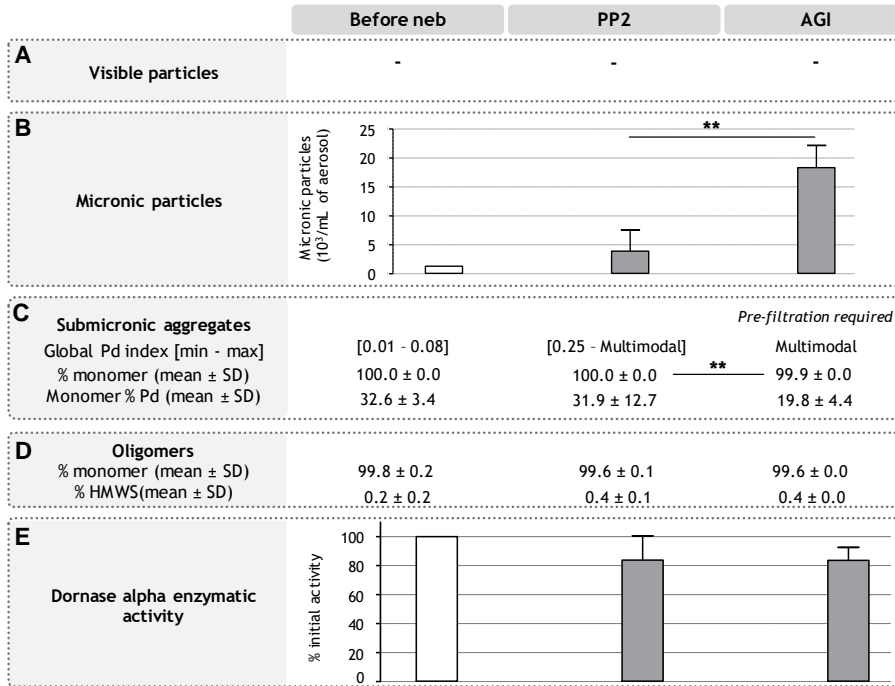
**Figure 4. Ig3 aggregation and antiviral activity after nebulization and collection with PP2 and AGI.**

Results are presented expressed as means ± one standard deviation between SD of the three replicates.

### 3.2.4. Comparison of PP2 and AGI on nebulized dornase alpha stability

Finally, we chose to include dornase alpha in this comparison, given its clinical relevance in the context of aerosoltherapy. After nebulization with an eFlow® rapid nebulizer at a concentration of 1 mg/mL, samples were collected with PP2 or AGI and characterized for aggregation and biological activity. As shown in Figures 5A and 5B, in spite of the absence of visible particles, a significant difference appeared for micronic aggregate concentrations, with a factor 4.7 between AGI and PP2 (18319 vs 3917 particles/mL, respectively). This discordance was also retrieved in DLS analyses (see Figure 5C): unlike PP2, AGI samples could not be analyzed without 0.45 µm pre-filtration, indicating multimodal distributions (which was confirmed by the global Pd index). After filtration, AGI samples had slightly lower monomer contents than PP2 samples. Even though their polydispersity tended to be reduced, these results suggest a greater loss of integrity for AGI samples. SEC analyses, performed after 0.2 µm filtration, showed quasi-identical profiles for the two aerosol samplers in terms of monomer and HMWS contents (see Figure 5D). For this protein, aerosol samplers had an impact on aggregation at the micrometric and nanometric scales.

From a biological point of view, we measured dornase alpha DNase activity by a colorimetric assay. Briefly, it quantifies the release of the dye methyl green from DNA upon enzymatic digestion by dornase alpha. Herein, the activity of the nebulized samples was expressed as a percentage of the activity of the initial solution. Results displayed in Figure 5E show that, even though nebulization caused a moderate decrease in enzymatic activity (-16%), the aerosol sampler had no impact on this parameter.



Pd: polydispersity; HMWS: higher molecular weight species

**Figure 5. Dornase alpha aggregation and enzymatic activity after nebulization and collection with PP2 and AGI.** Results are presented expressed as means ± one standard deviation between SD of the five replicates.

### 3.3. Synthesis: effect of the aerosol sampler on nebulized protein stability parameters

Table 5 summarizes the results obtained with each of the four studied protein therapeutics, in terms of concordance of aerosol samplers on protein stability parameters. For each parameter, we identified the sampler with the lowest impact on protein stability (*i.e.* with lower levels of aggregation or better preservation of biological activity). These data indicate a variable behavior of aerosol samplers, depending both on the protein and the stability parameter. For instance, concordance was observed for Ig3 on all parameters, whereas it happened only on biological activity for Ig1. Likewise, the impact of aerosol samplers on aggregation was heterogeneous, with inconstant concordance of the devices for the different particle populations. Finally, in discordant situations, the aerosol sampler with the lowest impact varied depending on the considered protein.



<b>Concordance between aerosol samplers?</b> (If not, sampler with the lowest impact on protein stability*)				
<b>Stability parameter</b>	<b>Ig1**</b>	<b>Ig2</b>	<b>Ig3</b>	<b>Dornase alpha</b>
<b>Visible particles</b>	No (AGI)	No (None)	Yes	Yes
<b>Micronic aggregates</b>	No (AGI)	Yes	Yes	No (PP2)
<b>Submicronic particles</b>	No (AGI)	Yes	Yes	No (PP2)
<b>Oligomers/soluble aggregates</b>	No (AGI)	No (PP2)	Yes	Yes
<b>Biological activity</b>	Yes	No (PP2)	Yes	Yes

\* Samplers with the lowest impact on stability parameters were identified based on the following criteria: absence of visible particles, lowest concentration in micronic aggregates, highest % monomer (for DLS and SEC) and highest biological activity.

\*\* For Ig1, results of device concordance were the same whether the comparison included 6 or 2 aerosol samplers.

**Table 5. Concordance between aerosol samplers on protein stability parameters.** This analysis included the 6 devices for Ig1, and two samplers (PP2 and AGI) for the other proteins. In case of discordance between aerosol samplers, the device with the lowest impact on protein stability is indicated in brackets.

#### 4. Discussion

When assessing nebulized protein stability, aerosol collection is a mandatory pre-analytical step; still, its interference on protein stability remains unknown. In the present study, we compared the effect of different aerosol samplers on the stability of four nebulized protein therapeutics.

First, we compared the devices in terms of aerosol collection. For Ig1, all devices gave similar collection efficiencies (60-75% of total Ig1 loaded in the nebulizer), except CYC, due to early saturation. However, PP2 and AGI displayed different collection performances depending on the protein therapeutics. On one hand, the concentration of Ig2 was notably affected by PP2 collection, with an apparent 2-fold dilution, which might result from non-specific protein adsorption on the plastic surfaces of the collection setup. On the other hand, AGI was associated with lower collection volumes for Ig2, maybe due to higher aerosol evaporation. In the end, when tested on four different proteins, these devices displayed variable collection performances, in terms of volume, protein concentration and protein amount. As aerosol characteristics were similar (regarding VMDs and nebulization output rates), these differences in aerosol collection might originate from specificities of protein molecules themselves, as could be the case with any "conventional" (non-biological) drug.

On the physicochemical side, protein aggregation was strongly affected by the aerosol collection device, with heterogeneous results for the four proteins. With Ig1, the six samplers gave six distinct aggregation profiles: micron-sized particle counts varied by a factor of 100, associated with variable monomer loss and inconstant presence of visible particles. For Ig2, the aerosol sampler rather impacted visible particles and oligomers, but not micronic particle counts. Conversely, after collection, dornase alpha displayed differences in micron-sized particles independently from similar profiles for oligomers and visible particles. The results for Ig3 are less conclusive because of the low initial protein concentration but tend to show similar aggregation profiles independently of the collection device. This heterogeneity of biotherapeutics aggregation due to the aerosol sampler is in agreement with Hertel, who observed that SM101 and IgG1 aggregation varied depending on the aerosol collection process [23]. Interestingly, in their previous study on dornase alpha aerosols, Cipolla and Gonda showed no difference in monomer loss after collection with two devices [22]. This discrepancy with our results may originate from differences in experimental conditions (the nebulizer and collection systems were different from ours) and incomplete characterization of protein aggregation.

Overall, the discordance in stability profiles might result from the different stresses applied to proteins during the collection process, originating from the underlying mechanisms of aerosol droplet recovery (*i.e.* condensation, impaction on solid or in liquid, vortex impaction). So far, it does not seem possible to conclude on a "more relevant" collection procedure, as

heterogeneities appeared for similar recovery mechanisms. For instance, AGI and NGI (which both imply impaction on a solid surface) yielded very different counts in micron-sized particles; likewise, PP15 and PP2, although very similar, gave distinct monomer/oligomer repartition. Other factors might be implied in these differences, such as air flowrate, material of the impaction surface or kinetic of droplet deposition.

Besides, in spite of similar collection efficiencies, the size range of collected droplets is not always known for each device. Although Ig1 distribution followed aerosol droplets distribution, indicating a homogeneous repartition in aerosol droplets (verified in a preliminary experiment, data not shown), one could imagine that some aerosol particles might be associated with higher levels of stresses. This hypothesis is supported by Ig1 aggregation results obtained with the NGI, for which the last plates (corresponding to smaller droplets) contained higher levels of visible and micron-sized particles than the others. This population of droplets has a higher specific surface area [4]; this implies that, for equal protein concentrations, a higher number of protein molecules is exposed to the deleterious air-liquid interface, as compared to larger droplets. Thus, fine droplets might be associated with more pronounced protein degradation, independent from collection stresses. In this case, large aggregates might probably appear after droplet deposition and coalescence, arising from interactions between highly altered protein species and/or nucleation of native monomers on the surface of aggregation nuclei [43].

In addition to aggregation, we also assessed the impact of the aerosol collection process on protein biological activity, which may change as a result of denaturation. Overall, aggregation was inconstantly associated with variations in protein biological activity. In spite of different aggregate populations obtained with AGI and PP2 collection, dornase alpha residual enzymatic activity was similar and well-conserved after nebulization, which is consistent with previous literature data [22]. Likewise, the binding of Ig1 to its target was not significantly affected by the nebulization/collection process; neither was Ig3 antiviral activity, in spite of higher variabilities observed with AGI. This is in agreement with current data on Ig nebulization, which was rarely reported to cause defects in target binding or cellular activity [12,14,39]. Interestingly, Ig2 behaved differently: its antibacterial activity was impacted by the collection system, as AGI samples were associated with higher rates of cytotoxicity. This effect seems to be linked rather to monomer-scale aggregation than to the absolute number of aggregates, as the amounts of micron-sized particles was similar for both devices. Additionally, these discrepancies in Ig functionality after nebulization might be explained by different aggregation patterns. Indeed, target recognition and binding (which were assessed for the three Igs) rely on small regions of Ig Fab portions. Thus, we could argue that for Ig1 and Ig3, aggregation affected other regions than the Fab, and hence was not critical for target binding (within the limit of sensitivity of the assays). Conversely, Ig2 aggregation might directly involve target-

binding regions, either through denaturation mechanisms or by simple entrapment and hiding of the antigen-recognizing site inside protein aggregates. Besides, for these Igs, aggregation might also impair Fc-dependent functions (such as FcγR binding or complement recruitment), which were not explored herein.

Taken together, these results highlight the variable impact of aerosol collection on protein stability. However, it seems difficult to discern general trends and identify the “most relevant” device for protein aerosols. Indeed, Ig1 aggregation and activity results tend to designate the AGI as the least deleterious sampler (with low levels of aggregation and no alteration of biological activity), while PP2 tubes were associated with higher levels of protein denaturation. Still, when tested on other proteins, AGI and PP2 gave opposite trends, PP2 being more favorable than the AGI for two (out of three) proteins, both from aggregation and activity prospects. Accordingly, Hertel and collaborators showed that PP2 tubes were associated with very low levels of particles and monomer loss of SM101, whereas the twin-stage impinger (which is close to the AGI) led to significant protein aggregation [23]. Therefore, the behavior of one protein with one sampler cannot be simply applied to others. Actually, this could be explained by the fact that proteins have different aggregation patterns, conditioned by their sequence, higher order structures, environment (formulation, concentration) and the stresses they face (e.g. nebulization stresses). Thus, depending on the considered protein, the “most relevant” aerosol sampler (i.e. the least deleterious one) varies, making it difficult to identify a universal collection method.

Besides, our findings raise the question of the relevance of such collection methods towards real-life considerations. Indeed, *in vivo*, droplet deposition sets up at 37°C, with 100% relative humidity, multidirectional air movements, on a large fluid-covered epithelial surface, and mainly implies a combination of inertial impaction, gravitational sedimentation and Brownian diffusion [46]. These conditions are very far from current *in vitro* aerosol collection processes, which probably induce significant biases on protein stability. For instance, with Ig2, both PP2 and AGI produced high amounts of particles > 25 μm, and even visible particles (> 500 μm). These species being larger than the biggest aerosol droplets (measured by laser diffraction), they were most probably generated after bulking of aerosol droplets, thereby favoring interaction between proteins from different droplets, regardless of the considered device.

Finally, this study allowed us to identify some of the criteria that would define an “ideal aerosol sampler” for protein therapeutics, namely suitability with any nebulization setup, representativeness of aerosol deposition in the lungs after inhalation and absence of interference on nebulized protein stability. Given that none of the tested devices fulfilled all these criteria, there is probably room for innovation in the field of aerosol collection for the development of inhaled protein therapeutics.

## **5. Conclusions**

This study highlights the fact that the aerosol collection step, which is mandatory to characterize nebulized proteins, has an impact on stability studies. More importantly, this impact depends on both the collection device and the protein, and it is thus hardly predictable for one given protein. To date, we did not identify a “universal” aerosol sampler, optimal for the stability of any protein therapeutic. Thus, careful selection and exclusive use of an appropriate aerosol sampler is crucial in the development of nebulized proteins, to limit pre-analytical variations and obtain comparable datasets. Until analytical characterization of proteins becomes possible in aerosol droplets, further optimization studies might allow to identify or develop a more relevant, maybe more physiological protein aerosol sampler.

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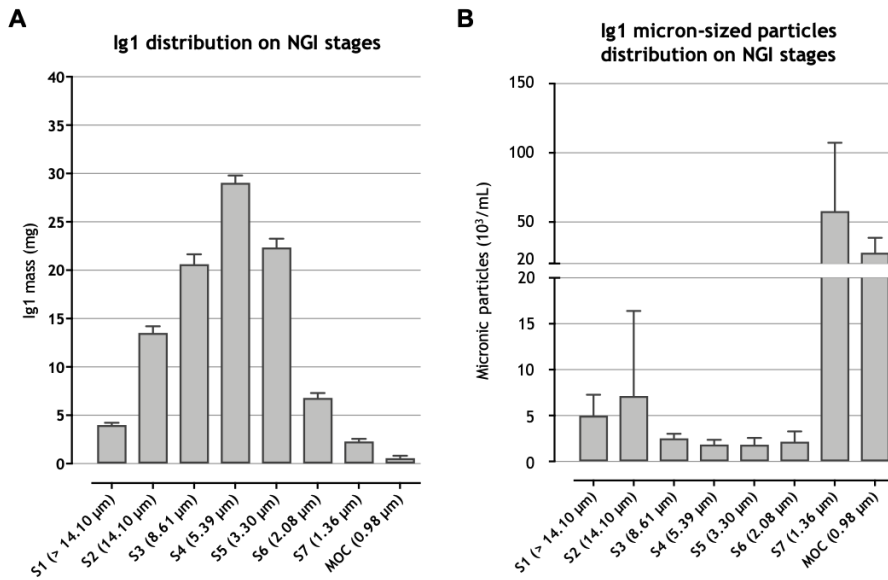
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## Supplementary data



**Figure S1. Ig1 deposition on NGI stages: protein and micron-sized distributions.**

**A.** Mass distribution of Ig1 on the NGI plates after mesh nebulization and collection. **B.** Concentrations in Ig1 aggregates  $\geq 1\mu\text{m}$  obtained by flow cell microscopy after Ig1 nebulization and deposition on the NGI plates. S1-S7 correspond to NGI stages 1-7, followed by the corresponding aerodynamic cutoff diameter in the present conditions of use. MOC: Micro-Orifice Collector.