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# **Designing inhaled protein therapeutics for topical lung delivery: what are the next steps?**

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## Abstract

Respiratory diseases (RDs) constitute a major public health issue worldwide, and some RDs represent a significant unmet medical need. Inhaled protein therapeutics are now attracting increasing interest for the topical treatment of RDs, with one drug on the market and almost 20 others in clinical development. Here, we review advances in inhaled protein therapeutics and comment on the development strategies that can be considered. To date, the transformation of a therapeutic protein into an inhalable drug has often been performed on a case-by-case basis by taking account of specific protein-, inhaler-, and patient-related factors. A rational design process for both the drug formulation and the delivery device is crucial for maintaining the protein's integrity and optimizing deposition in the lung, which in turn are decisive for clinically successful aerosol therapy. Despite significant advances in this field, there are still a number of outstanding questions about the stability of nebulized proteins and the consequences of nebulization on the biological activity, safety and pharmacokinetics of these innovative therapeutics. A better understanding of these parameters should promote the implementation of more rational development strategies, and thus accelerate progression towards clinical applications.

## 1. Advances in inhaled protein therapeutics

Respiratory diseases (RDs) constitute a major public health issue worldwide. Indeed, there are four RDs (pneumonia, tuberculosis, lung cancer, and chronic obstructive pulmonary disease (COPD)) among the ten most common causes of death. Additionally, RDs represent a major socio-economic burden through disability, healthcare costs, and loss of productivity.

In view of their advantageous pharmacological features, protein therapeutics (including antibodies) have ushered in a new era in respiratory medicine; they provide significant clinical benefit and are becoming increasingly important tools in the therapeutic armamentarium [1]. Given the limited diffusion of protein therapeutics from the bloodstream to the lungs (partly due to their high molecular weight), pulmonary delivery of aerosolized proteins seems an obvious choice - provided that the therapeutic target operates in the lungs, of course [2]. Approval of the first inhaled protein (the enzyme dornase alpha, marketed as Pulmozyme®) in 1993 reshaped the management of patients with cystic fibrosis; when combined with standard medications, the protein significantly improved lung function. After two decades, a number of promising new inhaled protein therapeutics with topical lung delivery are finally reaching the clinic – especially since 2010 (Table 1). To the best of our knowledge, 18 inhaled protein therapeutics have entered clinical trials and are being tested in either major RDs (asthma and pneumonia) or in rare/orphan lung diseases (e.g. cystic fibrosis, pulmonary alveolar proteinosis, and alpha-1 antitrypsin deficiency). It is noteworthy that two fatal RDs are missing from the list: lung cancer and COPD. Firstly, lung cancer is often detected late (i.e. with large, metastatic tumours), and inhaled protein therapeutics may not pass well into both the tumour tissue and the systemic circulation. However, *in situ* lung carcinoma with lepidic growth may be a relevant target for inhalation therapy [3]. Secondly, COPD is a complex disease with poorly elucidated underlying mechanisms and thus a lack of clear drug targets. To date, most protein therapeutics have countered infection-triggered acute exacerbations of COPD, which are correlated with disease progression and a decline in lung function. It is noteworthy that Synairgen has announced a Phase II trial of inhaled interferon beta (SNG001) to boost antiviral defences in patients with COPD.

## 2. Strategies for designing inhaled protein therapeutics

Although proteins can be formulated as dry powders for aerosolization, 75% of the inhaled protein therapeutics in clinical development have been developed as liquids for nebulization (Table 1). Accordingly, this editorial will mainly comment on advances in protein nebulization for topical lung delivery. Indeed, protein nebulization is often the first step in the formulation process because it offers several advantages over dry powders: it avoids drying steps, it is suitable for all clinical situations, and it enables greater pulmonary deposition than dry powder inhalers. Nebulizers have been significantly improved in recent years, and can deliver a 70% lung dose vs a maximum of 30% with current dry powder inhalers or the 50% expected with next-generation devices [4–6]. Indeed, dornase alpha is administered daily (2.5 mg per session) by nebulization. Nevertheless, nebulization exposes proteins to stressful conditions by generating a huge air-liquid interface (ALI) and, in some cases, high temperatures and/or shear forces. These conditions may cause protein unfolding, aggregation, oxidation, deamidation

or glycation, which may lead to changes in biological activity and safety concerns. In this prospect, nebulizers and formulations constitute two relevant tools to optimize protein stability during nebulization. Many marketed or investigational technologies have given encouraging preclinical or clinical results when used to aerosolize protein therapeutics (Table 2). A number of trends can be identified: as with other new inhaled therapeutics, vibrating-mesh nebulizers are now frequently used for therapeutic protein delivery in humans (86% of the cases in which the nebulizer technology is disclosed) [7]. Indeed, vibrating-mesh nebulizers allow the delivery of high drug amounts (often required for protein therapeutics) and are less harsh with regard to chemical and physical constraints [8]. For example, the ALX-0171 Nanobody™ aerosols displayed satisfactory aerodynamic properties and biologic potency when produced by vibrating-mesh nebulization, with no protein aggregation [9].

Formulation is also an important parameter in protein nebulization; it modulates the aerosol's aerodynamic profiles but also offers a unique opportunity to stabilize the protein. Since the mechanisms underlying protein degradation during nebulization are not always well understood, formulation design often relies on an empirical screening of excipients already known to stabilize proteins and/or improve aerosols. To prevent aggregation (occurring mainly at the ALI), surfactants (e.g. polysorbates) have been successfully included in formulations of monoclonal antibodies and aviscumine [10,11]. Polyethylene glycols have also been used to stabilize proteins by generating steric repulsion between monomers [10]. Likewise, protein therapeutics can be stabilized by other excipients (buffers, sugars, polyols and amino acids, Table 1). However, due to intellectual property concerns, formulation details are rarely disclosed for inhaled proteins in clinical development (i.e. for only six proteins in Table 1). This clearly limits the extrapolation of general principles to the nebulization of protein therapeutics.

Formulation may also make it possible to enhance a protein's pharmacological properties. For instance, modified-release formulations (such as microparticles, nanoparticles or liposomes) might customize pharmacokinetic profiles while avoiding rapid protein degradation [12]. Nevertheless, few FDA-approved excipients for inhalation (i.e. endogenous lung compounds or inactive formulation ingredients) are currently available. Thus, progress in formulation advances may require the safety of new excipients to be assessed.

### 3. Expert opinion

Nebulization is now a leading approach for administering proteins to the airways, as evidenced by the number of ongoing clinical trials in this field. Today's success stories highlight the advantages and potential of nebulization for topical lung delivery - as long as the inhalation system ensures protein stability and effective drug deposition within the respiratory tract. However, a number of molecule- and/or administration-route-related challenges remain (Figure 1).

At present, there are several relevant device and formulation options for effective, safe protein aerosolization. The significant interplay between these two parameters has prompted the development of unique drug-device combinations tailored to the protein of interest (in which the device and the formulation are engineered in tandem). Indeed, four of the inhaled proteins in clinical trials are delivered with a customized nebulizer (Table 1). However, redesigning the device and the formulation might not be feasible or relevant for every new protein. If rapid development is required or if the protein is only

moderately sensitive to aerosolization stresses, it may be more straightforward and affordable to adjust the formulation to a marketed device.

One of the particularities of the inhaled route relates to the mechanisms of protein degradation, which are still partially elucidated. Although all proteins will be exposed to a large ALI (the magnitude of which depends on the droplet size), the level of applied stress probably differs from one device to another. For example, aerosolization with two different mesh nebulizers generated distinct aggregate populations with the same protein [11]. Hence, aerosol development approaches might need to be reconsidered for each new protein. Gaining insight into the physical/chemical mechanisms contributing to protein degradation will be crucial for protecting the active compound and making formulation design more rational and time-efficient.

It is still not clear how to assess protein stability. Although aggregation and biological activity are common read-outs, other protein alterations (e.g. oxidation or deamidation) are less frequently studied - despite their obvious links to protein activity and immunogenicity [13]. In the literature, a range of analytical techniques are employed to assess protein stability and aggregation, with various levels of performance. This complicates inter-study comparisons and the ability to highlight key parameters in protein stability. In addition, most of today's analytical methods are not applicable to aerosol droplets. Hence, droplets must be pooled into a bulk liquid before analysis, which might modify protein stability and thus induce pre-analytical bias. Hence, there is a need for in-depth, relevant assessments of the physicochemical and biological aspects of protein stability following aerosolization.

Given the specific characteristics of the inhalation route, dedicated studies of aerosol-specific stresses, subsequent protein modifications, and their impacts on efficacy, pharmacokinetics and safety are required. For example, immunogenicity appears to vary markedly among the four proteins studied to date, and is difficult to predict (Table 1). As evidenced by several *in vivo* studies, the protein aggregates formed in response to various stresses may raise immunogenicity, and prompt the production of potentially neutralizing anti-drug antibodies. This issue has not yet been well addressed for aerosolization stress and airway delivery.

The use of animal models to analyze the efficacy, pharmacokinetics and safety of inhaled drugs has been informative but remains cumbersome and ethically problematic. Indeed, it is difficult to extrapolate animal data on inhaled protein therapeutics to humans because of physiological, anatomical, biological and respiratory differences between species; the use of dissimilar aerosol devices induces distinct stresses on protein therapeutics and unequal drug deposition within the respiratory tract [14]. The recent implementation of promising *in vitro* models (such as multilayer cell-based models in an aerosol chamber) may simplify inhaled drug development by integrating advanced functional studies in the early formulation steps [15]. Aerosol delivery in complex organ-mimicking systems (such as "lungs-on-a-chip") might also generate other options [16].

In conclusion, the development of proteins for nebulization is eminently possible, in view of the current state of scientific knowledge and recent successes in this field. However, addressing unresolved questions about protein stability (Figure 1) might help to standardize the development processes, facilitate the elaboration of dedicated regulatory guidelines, and accelerate progression towards clinical applications. Some of these concerns are also relevant for the development of dry protein aerosols; as

already observed for anti-infectives, the use of these dry inhaled formulations is expected to increase over the coming years and decades.

Protein therapeutic		Clinical development				Inhalation system		Immunoge nicity (ADAs)	Reference / Clinical trial # (NCT)* / Patent # (EP/WO)
Name(s)	Molecular characteristics	Companies / Sponsors	Stage	Therapeutic indication	Dosing regimen	Formulation	Inhaler		
Marketed									
Dornase alpha	Deoxyribonuclease I (37 kDa)	Roche	Marketed	Cystic fibrosis	2.5 mg/d	C =1 mg/mL Exc: sodium chloride, calcium chloride dehydrate (pH 6.3)	Jet or mesh nebulizers	ADAs in <5% of patients (n=643)	Pulmozyme® SmPC [17]
Under clinical evaluation									
ALX-0171 / VR465	Trivalent Nanobody™ (42 kDa) targets RSV F-protein	Ablynx	Phase IIb ongoing	RSV infection	3 different doses Once daily x 3 d	C = 50 mg/mL Exc: sodium chloride, phosphate buffer (among others)	Mesh nebulizer (customized Vectura FOX-Flamingo® system)	ADAs in 23% of patients (n=30)	NCT02979431 [9,18]
VR942 / UCB4144 / CDP7766	Anti-IL-13 mAb fragment	UCB Pharma	Phase I completed (2016)	Asthma	0.5-20 mg, single- or repeated dose (10 d)	N/A	DPI	No clinical evidence of immunogeni city	NCT02473939 [19]
Alpha-1 antitrypsin (Kamada-API)	Lung serine protease inhibitor (52 kDa)	Kamada	Phase II/III (Eur) completed (2015)	Alpha-1 antitrypsin deficiency	N/A	C = 20 mg/mL	Mesh nebulizer: customized PARI eFlow®	N/A	NCT01217671 [20]
			Phase II (US) completed (2015)	Alpha-1 antitrypsin deficiency	80-160 mg/d	N/A	Mesh nebulizer: customized PARI eFlow®	N/A	NCT02001688
Alpha-1 antitrypsin (Alpha-1 HC)	Lung serine protease inhibitor (52 kDa)	Grifols Therapeutics	Phase II completed (2013)	Cystic fibrosis	100-200 mg/d x 21d	C = 50 mg/mL Exc: PBS, polysorbate	Nebulizer	N/A	NCT01684410
GSK2862277	Anti-TNF-R1 mAb V <sub>H</sub> domain (13 kDa)	GSK	Phase II terminated (2018)	Acute lung injury	26 mg, single dose	Lyophilizate, reconstitution before nebulization Exc (Phase I trial): sucrose, glycine, sodium dihydrogen phosphate, polysorbate 80	Mesh nebulizer (PARI eFlow® with s30 mesh)	N/A	NCT02221037 [21]
AIR DNase™ / Alidornase alfa / PRX-110	Actin-inhibition- resistant deoxyribonuclease I (37 kDa)	Protalix BioTherapeutics	Phase I competed (2016)	Cystic fibrosis	1.25 – 5 mg/d x 5 d	N/A	N/A	N/A	NCT02605590
			Phase II ongoing	Cystic fibrosis	2.5 mg/d x 28 d	N/A	Mesh nebulizer (Philips I-neb AAD®)	N/A	NCT02722122

<b>Alteplase</b>	Tissue plasminogen activator (tPA) (70 kDa)	University of Michigan, Genentech	Phase II ongoing	Plastic bronchitis	5 mg every 6h x 3 d	Exc (Activase®): arginine, phosphoric acid, polysorbate 80	Nebulizer	N/A	NCT02315898
<b>ALX-009: lactoferrin</b> (+ hypothyocyanite)	Antibacterial glycoprotein (80 kDa)	Alaxia Pharma	Phase I ongoing	Cystic fibrosis	Single / multiple administrations	C = 4 mg/mL	Nebulizer	N/A	NCT02598999
<b>Molgramostim</b>	Recombinant human GM-CSF (14 kDa)	University of Giessen	Phase II ongoing	ARDS	150-450 µg/d x 3 d	N/A	N/A	N/A	NCT02595060
		Savara Pharmaceuticals	Phase II / Phase III ongoing	APAP	300 µg/d x 24 w continuously or intermittently	N/A	Mesh nebulizer (investigational PARI eFlow®)	N/A	NCT02702180 NCT03482752
			Phase IIa ongoing	NTM infections	300 µg/d x 24 w	N/A	Mesh nebulizer (investigational PARI eFlow®)	N/A	NCT03421743
<b>Sargramostim (Leukine®)</b>	Recombinant human GM-CSF (14 kDa)	Milton S. Hershey Medical Center	Phase I ongoing	Paediatric ARDS	25-250 µg/m² x 7 d	Exc (Leukine®): mannitol, sucrose, tromethamine ± benzylalcohol	Nebulizer	N/A	NCT02601365 Leukine® PI
		Children's Hospital Medical Center, (Cincinnati), Genzyme	Phase II completed (2014)	aPAP	250 µg/w*	Exc (Leukine®): mannitol, sucrose, tromethamine ± benzylalcohol	Nebulizer	N/A	NCT01511068 Leukine® PI
		Children's Hospital Medical Center (Cincinnati)	Phase I ongoing	aPAP	125-250 µg, single dose	Exc (Leukine®): mannitol, sucrose, tromethamine ± benzylalcohol	N/A	N/A	NCT03006146 Leukine® PI
		IRCCS Policlinico S. Matteo	Phase II/III (status unknown)	aPAP	250 µg/d*	Exc (Leukine®): mannitol, sucrose, tromethamine ± benzylalcohol	N/A	N/A	NCT00901511 Leukine® PI
		Barbara Ann Karmanos Cancer Institute	Phase II completed (2010)	Lung metastases / cancer	250 µg bid*	Exc (Leukine®): mannitol, sucrose, tromethamine ± benzylalcohol	N/A	N/A	NCT00514215 Leukine® PI
		Children's Oncology Group	Phase II completed (2013)	Osteosarcoma with lung spreading	240-1750 µg bid*	Exc (Leukine®): mannitol, sucrose, tromethamine ± benzylalcohol	N/A	N/A	NCT00066365 Arndt 2010 Leukine® PI
<b>GM-CSF</b>	Recombinant human GM-CSF (14 kDa)	Niigata University Medical & Dental Hospital	Phase II completed (2017)	aPAP	125 µg bid*	N/A	N/A	N/A	NCT02835742

		Peking Union Medical College Hospital	Phase II (status unknown)	aPAP	150 µg bid*	N/A	Nebulizer	N/A	NCT02243228
		Dai Huaping	Phase II ongoing	aPAP	150 µg bid*	N/A	N/A	N/A	NCT03316651
<b>SNG001</b>	Interferon beta-1a (22 kDa)	Synairgen	Phase II completed (2012)	Asthma	6 mL/d x 14 d	Neutral pH, no mannitol, no human serum albumin	Mesh nebulizer (Philips I-neb®)	N/A	NCT01126177 [22]
		Synairgen, AstraZeneca	Phase II completed (2016)	Asthma	24 µg /d x 14 d	C = 48 µg/mL	Mesh nebulizer (Philips I-neb®)	N/A	NCT02491684
<b>AZD1402 / PRS-060</b>	Anticalin protein (~18 kDa) Target: IL-4Rα	Pieris Pharmaceuticals, AstraZeneca	Phase I ongoing	Asthma	Single dose	N/A	Nebulizer	N/A	NCT03384290 [23]
<b>DAS181 (Fludase®)</b>	Recombinant neuraminidase (46 kDa)	Ansun Biopharma	Phase II	Influenza virus infection	10 mg/d x 3 d	N/A (named F02)	DPI	N/A	NCT01037205 EP2841092A1
		National Institute of Allergy and Infectious Diseases (NIAID)	Phase I completed (2011)	Influenza virus infection	20-30 mg single dose or 20 mg/d x 10 d	C = 70% (w/w) Exc: histidine, trehalose, citric acid, acetate buffer (named F03)	DPI	IgA or IgG in 88% of patients (n=18)	NCT01173224 [24]
			Phase I completed (2012)	Influenza virus infection	20 mg/d x 3d	C = 65% (w/w) Exc: histidine, trehalose, MgSO <sub>4</sub> , citric acid, acetate buffer (named F04)	DPI		NCT01651494 [24]
<b>DAS181 (Paradase®)</b>	Recombinant neuraminidase (46 kDa)	Ansun BioPharma	Phase II completed (2017)	Parainfluenza virus infection	DPI: 10 mg/d x 7 d Nebulizer: 3.2-4.5 mg/d x 7 d (maximum total dose = 28.9 mg)	DPI: F02 (undefined) Nebulization: F02 dissolved in 10 mL normal saline	DPI (Teva Cyclohaler®) Nebulizer	N/A	NCT01924793 [25]
			Phase II completed (2016)	Parainfluenza virus infection	4.5 mg/d x 10 d	F02 (undefined)	DPI	N/A	NCT01644877
<b>CSJ117</b>	Anti-TSLP mAb fragment (46 kDa)	Novartis	Phase I ongoing	Asthma	3 different doses 1 dose/d x 12 w	Exc: from among leucine, trileucine, HCl, trehalose, mannitol. Buffers: from among histidine and glycine	N/A	N/A	NCT03138811 WO2017042701A1

<b>AER-001 / BAY 16-9996 / Pitrakinra / Aerovant®</b>	rhIL-4 (14 kDa) IL-13R and IL-4R antagonist	Aerovance, Bayer	Phase II completed (2006)	Asthma	60 mg bid x 28 d	N/A	Jet nebulizer (PARI LC® Plus)	N/A	NCT00535431
		Aerovance, Bayer	Phase II completed (2010)	Asthma	1-10 mg bid	N/A	DPI	N/A	NCT00801853 [26]
<b>rhIL-2</b>	Recombinant cytokine 15 kDa	MD Anderson Cancer Center	Phase I/II ongoing	Sarcomas, lung metastases	1 mg/d x 21 d	C = 0.33 mg/mL Exc (Proleukin®): mannitol, sodium laurylsulphate, phosphate buffer	Nebulizer	N/A	NCT01590069
<b>Stopped / Inactive</b>									
<b>IFN-γ 1b</b>	Interferon gamma-1b	InterMune, NIAID	Inactive	MAC infections, cystic fibrosis	500-1000 µg x 3/w	N/A	N/A	N/A	NCT00043355 NCT00021567 NCT00043316
<b>Omalizumab</b>	Anti-IgE IgG1 (150 kDa)	Genentech / Novartis	Stopped	Asthma	1-10 mg/d x 8w	N/A	Jet nebulizer (PARI IS-2)	IgG in 5% patients (n=22)	[27]
<b>Alpha-1 antitrypsin</b>	Lung serine protease inhibitor (52 kDa)	Kamada,	Phase II completed (2008)	Cystic fibrosis	80 mg/kg x 7-28 d	N/A	Mesh nebulizer (customized PARI eFlow®)	N/A	NCT00499837

**Abbreviations:** ADA, anti-drug antibody; mAb, monoclonal antibody; Fab, antigen-binding fragment; RSV, respiratory syncytial virus; DPI, dry powder inhaler; mg/d, milligrams per day; PBS, phosphate buffer saline; TNF-R1, tumor necrosis factor-receptor 1; ARDS, acute respiratory distress syndrome; w, weeks; aPAP, autoimmune pulmonary alveolar proteinosis; BID, *bis in die*; NTM, nontuberculous Mycobacterium; C, protein concentration in the final product; Exc, excipients; GM-CSF, granulocyte-macrophage colony-stimulating factor; MAC, Mycobacterium avium complex; NIAID, National Institute of Allergy and Infectious Diseases; SmPC, summary of product characteristics; PI: prescribing information.

\* Clinical trial numbers refer to the latest clinical trials registered at [clinicaltrials.gov](http://clinicaltrials.gov)

N/A: not available

**Methodology:** clinical trials involving inhaled protein therapeutics for topical lung applications were identified using the [clinicaltrials.gov](http://clinicaltrials.gov) database. The following research filters were applied: study type: interventional studies; conditions: respiratory tract (lung and bronchial) diseases.

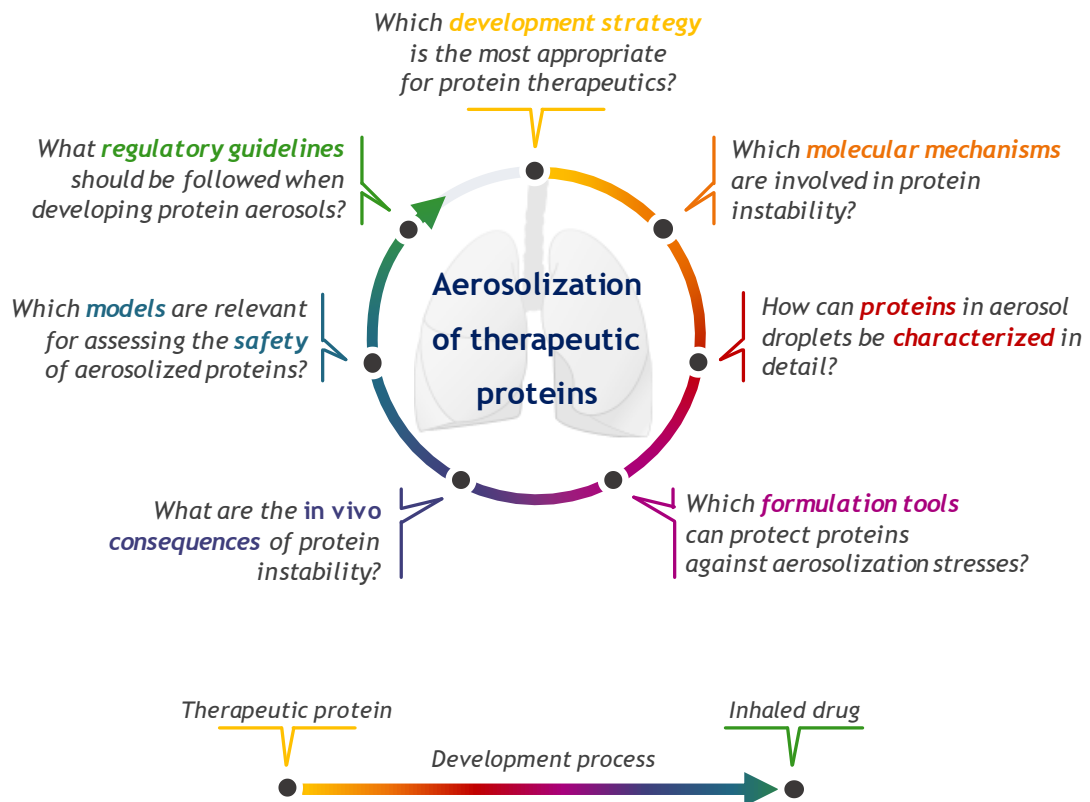
**Table 1.** Inhaled protein therapeutics in clinical development for topical lung applications

Inhaler technology	Precise, reproducible dosing	Compatibility with high doses	Minimal drug loss, and high lung deposition	Maintenance of protein integrity	Limitations/remarks	Reference
<b>Marketed nebulizers</b>						
Jet nebulizers					Device performance affected by the drug solution's physicochemical properties (viscosity, surface tension, etc.)	[8,10]
Ultrasonic nebulizers						
Mesh nebulizers						
Soft mist inhalers					Relatively unsuitable with dispersed systems	
<b>Emerging/investigational devices with at least one feasibility study reported for proteins</b>						
Aradigm AERx® (slow-velocity mist)					Aerosolization profile affected by the drug solution's properties	[28]
Surface acoustic wave (SAW) devices	Not available				Lack of <i>in vivo</i> data	[28,29]
<b>Legend</b>						

**Trends are based on our best knowledge of literature reports.**

NB: Staccato™ technology was not included in this table, given its incompatibility with peptides and proteins.

**Table 2.** Desirable characteristics for protein inhalers, and trends in nebulization devices.



**Figure 1.** The development of nebulized protein formulations: several key questions have yet to be addressed.

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- **Extensive state-of-the art of protein nebulization and stability concerns. (Hertel)**
- **Overview of liquid aerosolization devices, covering marketed to investigational stages. (Carvalho)**
- **Full development strategy of an inhaled protein, from molecular targeting to clinical evaluation. (Van Heeke)**
- **Overview of the possibilities offered by formulation to enhance inhaled drugs pharmacokinetics. (Loira-Pastoriza)**
- **A novel cell-based tool to optimize the development of inhaled proteins, especially formulation screening. (Röhm)**