Device and formulation considerations for nebulized biologics

Limiting the impacts of aerosol generation on biotherapeutics and delivery systems

Matthew J. Owen, PhD; David Wilcox, BS and Alan B. Watts, PhD
Catalent Pharma Solutions

Introduction

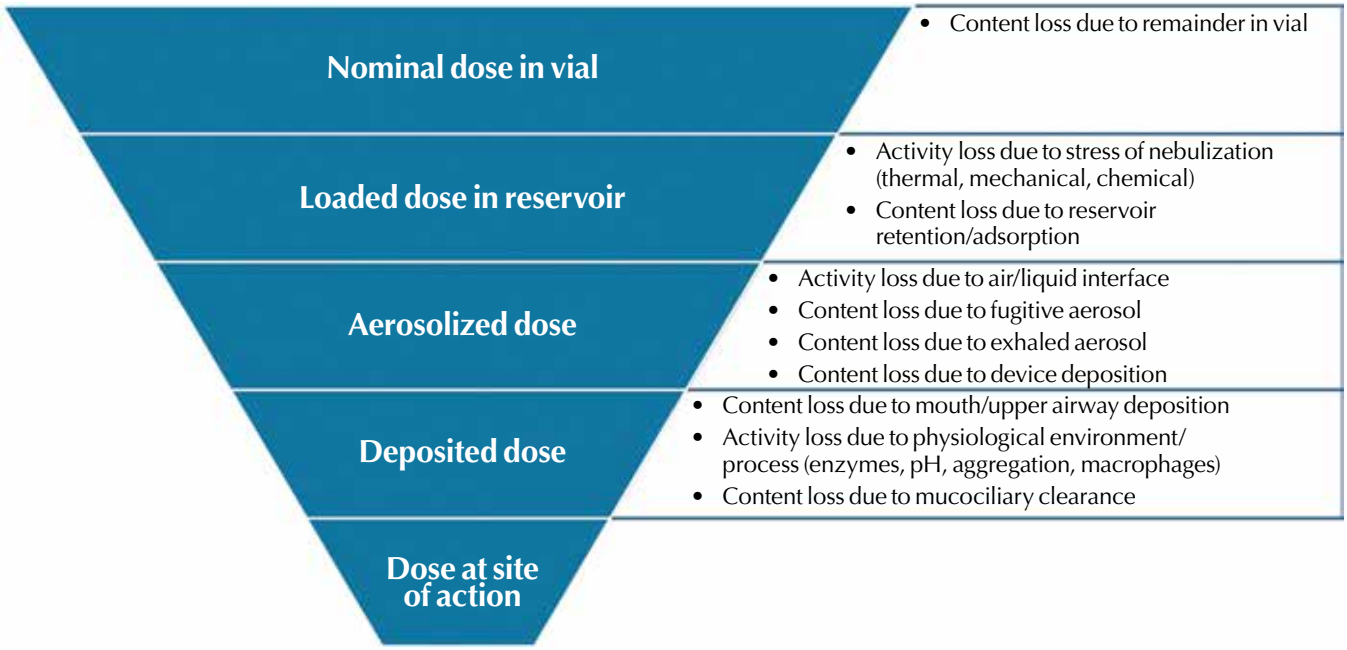
The number of new biotherapeutics for inhalation is increasing rapidly. A number of pipelines, many of which are within small to mid-size biopharmaceutical companies, contain biologics targeting a variety of pulmonary diseases. These include viral or bacterial infection, genetic diseases such as cystic fibrosis (CF) or primary ciliary dyskinesia (PCD), fibrotic diseases like idiopathic pulmonary fibrosis (IPF) or historically targeted acute and chronic inflammatory diseases such as asthma and chronic obstructive pulmonary disease (COPD). Although there are substantial challenges to delivering biologic therapies to the lungs (to be discussed in this article), the respiratory pathway does provide the benefit of a large surface area for absorption that is highly vascularized with reduced metabolic, enzymatic and pH extremes in comparison to the oral route. The reduced harshness of this environment is beneficial for labile biologic therapies, making it more likely that they reach their site of action fully intact and potent. The vast majority of inhaled biologics in development are focused on local disease, although there is potential to utilize respiratory delivery for systemic delivery of biopharmaceuticals. When compared to injection, the pulmonary route offers a needle-free alternative to self-administer a biotherapeutic. Inhaled insulin was the first commercialized product to demonstrate the utility of the pulmonary route for systemic therapy, however to date, the "lung to systemic" strategy has been largely unutilized. While having a smaller surface area than the lungs, the nasal passages are being more often used for systemic therapy and vaccination, likely due to less restrictive and potentially less degrading aerosol size requirements, a wider variety of approved excipients and reduced concern for local inflammation and tolerability compared to the pulmonary route [1]. Nasal delivery of biologics is of growing interest, particularly for targeting central nervous system (CNS) diseases [2], but for purposes of this article, we will focus on the delivery of biotherapeutics via nebulized liquids intended for pulmonary deposition.

We will consider device and formulation approaches for inhaled biologics and conclude with a case study that investigates the influence of nebulization on polyA-loaded lipid nanoparticle (LNP) integrity. We use the term "biologics" to cover a very heterogenous class of compounds and delivery systems that are being investigated for pulmonary delivery. These include two primary classes of biologic compounds: 1) peptides and proteins, which include synthetic peptides, recombinant proteins, enzymes, antibodies and antibody fragments and 2) nucleic acids, which include ribonucleic (RNA), LNP and polymeric encapsulated RNA, antisense oligonucleotides, and adeno-associated virus (AAV) vector gene therapy, among others [3].

There are other classes of biologic therapies, which would require an independent "other" category. These include bacteriophages, cellular or stem cell therapy and attenuated viruses for vaccination. Many of the same stratigies to preserve active stability and potency outlined below would apply to these "other" therapies as well, however, there may be additional considerations. Those readers interested in these forms of advanced inhalation therapy are encouraged to see reviews on inhaled phages [4], stem cells [5] and mucosal vaccination via the respiratory tract [6].

Figure 1

Loss of active drug substance throughout the process of dosing a liquid aerosol to the lungs.



Device and formulation effects on stability

Liquid aerosols intended for pulmonary delivery are defined by their small droplet size (1-5 µm), resulting in an extremely large liquid surface area of up to 1,500 m^2 [7]. While fine droplets are essential for lung deposition, proteins will migrate toward the air/liquid interface of the droplet where they can aggregate and/or unfold and denature. Other degrading forces seen during aerosol generation can include mechanical/vibrational, thermal and chemical stress. Chemical stress of a biologic is often manifested as oxidation, hydrolysis or deamidation and may be traced back to chemical impurities in drug substance or excipient, pH shifts or an initial physical stressor. An understanding and control of instability and aggregate formation is critical, not only because it reduces the therapeutic activity of the protein formulation, but because aggregates may elicit immunogenicity *in vivo*.

Delivery of a liquid biologic aerosol to the lungs is a complex endeavour with many opportunities for activity loss. Figure 1 illustrates losses at subsequent stages of nebulization due to degradation or simply loss of content as the biotherapeutic makes its way from the primary container closure to the site of action. Appropriate device selection and formulation can help mitigate some of these losses, and equally importantly, provide consistent and predictable delivery.

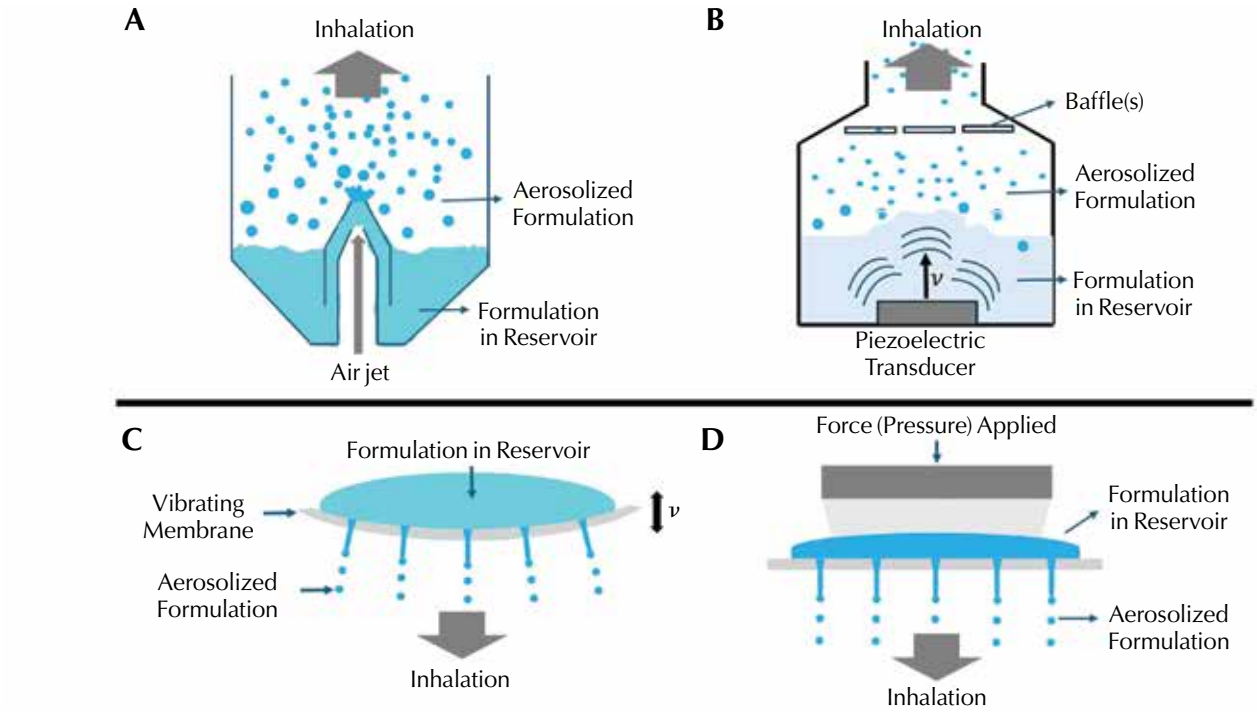
Device considerations

There are a variety of devices available commercially and in development to generate liquid aerosols for pulmonary delivery, many of which have been utilized for delivery of biologic compounds. The mechanisms of these devices are depicted in Figure 2. These have common, as well as unique, attributes that can impact the active pharmaceutical ingredient's (API's) stability or encapsulated/vectored particle integrity.

Air jet nebulizers are among the oldest and most established devices used to produce a liquid aerosol. Their basic function relies on the Bernoulli principle, where air flow creates a negative pressure that draws liquid from a reservoir through a small capillary where it then is atomized by an air flow jet. The atomized liquid then impacts baffles within the nebulizer that remove larger, non-respirable droplets and allows them to drain back into the reservoir. While appropriate for small molecules, this recirculation of liquid impacted on baffles can contribute to protein denaturation and increase the likelihood of aggregate formation. Additionally, jet nebulizers can be inefficient in delivery (generally having a low output rate), produce fugitive aerosol during exhalation and leave residual non-aerosolized product in the reservoir. They do not, however, utilize any heat-generating vibrational energy, which has been shown to contribute to protein instability [8]. Further, fugitive aerosols can be largely mitigated by utilization of breath-enhanced or breath-actuated air jet nebulizers [9]. While air jet nebulization has been found suitable for delivery of more robust proteins such as dornase alpha [10], low output rates, recycling of aerosol within the device and limited product-specific tun-

Figure 2

Functional mechanisms for types of nebulizers: a) Air jet, b) Ultrasonic, c) Vibrating mesh and d) Soft mist.



ability present challenges when utilizing these devices for more complex and labile payloads.

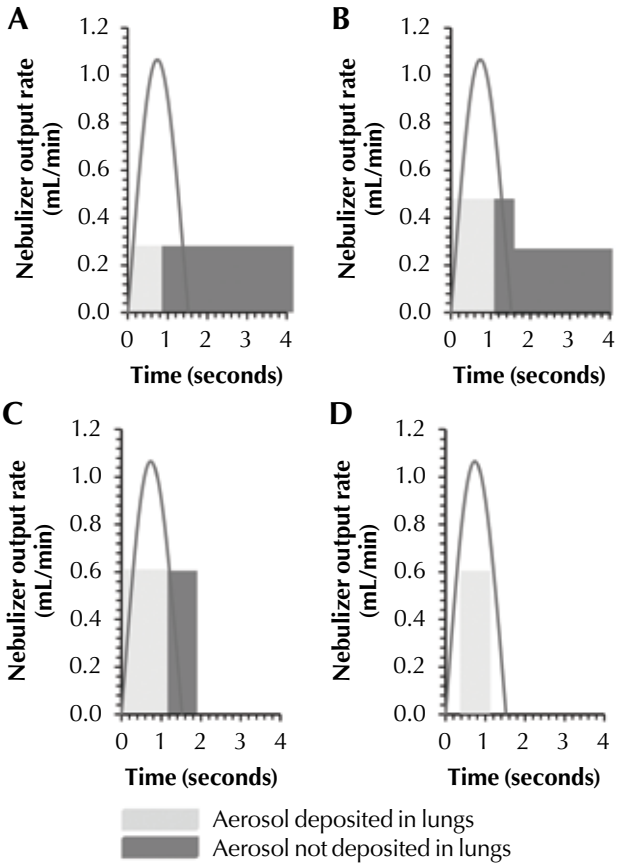
Ultrasonic nebulizers operate by passing acoustic waves generated by a vibrating piezoelectric transducer through a volume of liquid. The high frequency vibrations (approximately 1-3 MHz [11]) cause cavitation within the liquid—where small vapor bubbles form and collapse, resulting in the formation of aerosol droplets. The ultrasonic transducer generates heat during operation, which can pass through the liquid formulation and degrade heat-sensitive biologic medications. In some instances, temperatures have been shown to increase above 50 °C in the liquid reservoir [12]. Additionally, ultrasonic nebulizers may struggle to aerosolize highly viscous biologic formulations. These factors represent substantial hurdles for use of ultrasonic nebulizers with biologics.

Vibrating mesh nebulizers (VMN) are utilized in several development programs and clinical pipelines for delivering inhaled peptides, proteins and nucleic acids. They utilize a mesh or perforated plate containing thousands of microscopic holes (also called bores or apertures) to create aerosolized droplets from liquid formulations. The most common type are active VMNs, where the mesh itself vibrates at high frequencies (100-150 kHz) [13] to pump liquid through the apertures. However, there are passive VMNs where the mesh is static and a transducer next to the mesh provides the acoustic energy to move liquid through the mesh. Mesh technology allows adaptation of variables like hole size and shape, membrane properties, and vibration frequency and amplitude to reduce the degrading impact on sensitive formulations. Electronic control of the membrane also allows for synchronization of aerosol generation with patient breathing pattern for more efficient delivery compared to conventional continuous nebulization [14]. Figure 3 illustrates how generating an aerosol only during a small portion of the inspiratory maneuver can result in the most efficient delivery to the lung periphery, reducing wasted/fugitive aerosols. Vibrating mesh devices typically have output rates up to 0.7 mL per minute, generally higher than jet nebulizers (0.2 mL/min)[15], and negligible residual waste in the reservoir (~ 0.1 mL). Heat can be generated by VMNs depending on device and formulation characteristics, however not to the extent of an ultrasonic nebulizer. In a study of aerosolized recombinant human deoxyribonuclease (rhDNase), reservoir temperatures at the end of ultrasonic nebulization reached 58 °C [12], while temperatures in a vibrating mesh nebulizer have been found to reach only 40 °C [16]. The mesh component of a VMN can become clogged with particulate matter such as aggregated protein or lipid, which may impair nebulization performance. Thorough study of use and misuse, which may lead to clogging as well as mitigations such as cleaning or mesh replacement, should be undertaken, particularly for high concentration formulations and LNP-based products.

A soft mist inhaler (SMI) can broadly be categorized a propellant-free, aqueous, aerosol-generating device that uses mechanical energy to extrude a liquid formulation through small channels to generate a respirable mist. Colliding and Rayliegh jets are the two

Figure 3

Nebulization approaches to synchronize aerosol delivery with breathing waveform; where d > c > b > a for efficiency in targeting the lung periphery. a) Conventional air jet, b) Active Venturi air jet, c) Breath-synchronized and d) Targeted-breath synchronization. Reproduced from Reference 14.



principles by which these slow-moving inhalable mists can be generated. These mechanisms of aerosol generation do not involve any high-flow compressed air or piezoelectric vibration, utilizing only a static block or mesh through which liquid is extruded. The commercially available Respimat® (Boehringer Ingelheim, am Rhein, Germany) utilizes twin colliding jets to deliver beta agonist and anti-muscarinic therapies for asthma and COPD patients. These therapies require very low volumes of liquid to be inhaled (15 µL) however, other SMIs have demonstrated the ability to delivery up to 1 mL over ~ 2 min [17]. For delivery of proteins or lipid nanoparticles that are sensitive to shear stresses, SMIs have been shown capable of minimizing impact on LNPs [18], perhaps due to the absence of a vibrating piezo element. Similar to VMNs, SMIs may be prone to clogging when delivering suspensions or high concentration formulations where aggregation may occur. Capability for multiple doses within a single device can be advantageous from a convenience perspective but presents a challenge in ensuring sterility over the life of the product. Unlike pressurized metered dose inhalers (pMDIs), where propellants would not promote bacterial growth, SMIs are either completely or nearly completely formulated in aqueous media.

Standing acoustic wave (SAW) nebulization is a recently developed technology for aerosol generation. Through utilization of nanometer amplitude acoustic waves and high frequencies, SAW nebulizers can nebulize single a droplet of fluid into a respirable aerosol. Energies used for nebulization have been shown to be an order of magnitude less than those used in ultrasonic nebulization, with 0.5-3 W used in SAW nebulizers compared to 10 W or more in ultrasonic nebulizers [19]. These low energies have been shown capable of generating a respirable aerosol of small interfering ribonucleic acid (siRNA) with minimal damage, likely due to reduced cavitation and shear force [20]. Further, naked plasmid DNA was shown to be successfully aerosolized with more than 90% remaining intact [21]. While SAW nebulization shows promise for delivery of naked nucleic acid formulations, more investigation is needed for evaluation of high viscosity and suspension-based biologic formulations.

Formulation strategy

Along with device selection and screening, formulation is critically important when delivering a liquid biologic aerosol. Unlike many small molecule intravenous (IV) formulations that can be suitable for nebulization without reformulation, more labile biologics formulated for IV administration may not effectively withstand the added stress of nebulization. Additionally, physicochemical properties such as viscosity, surface tension and ionic strength have been shown to affect the ability to nebulize, the aerosol delivery rate and the resulting droplet size distribution [9]. In particular, viscosity can be greatly affected by high concentrations of protein, nucleic acid and nanoparticulate dispersions as well as by polymeric surfactants. Non-ionic surfactants such as polysorbate, poloxamer and polyethlyene glycol have all been utilized in nebulized products to limit aggregation during nebulization and prevent surface absorption to the device and container closure [22]. Polysorbate 20 and 80 at concentrations of up to 0.1% w/v have been the most utilized for nebulized formulations, however oxidative stress due to trace peroxides in these excipients can promote instability over time and should be accounted for in raw material testing. These reactive substances can impact stability in both vialed formulation and after nebulization. Another formulation has found that use of albumin had a stabilizing effect on a liquid recombinant protein formulation, perhaps as an oxygen scavenger [22]. Novel surfactants have been designed to improve stability of protein formulations while limiting toxicity through modification of a well toler-

ated sugar, trehalose, by adding a fatty acid chain and an ionizable succinate. *In vitro* and preclinical results suggest this trehalose-based excipient can effectively stabilize a monoclonal antibody (mAb) for treatment of COVID infection by nebulization [23].

Buffering systems also, clearly, play a role in stabilization of a biologic formulation by maintaining the liquid formulation at a suitable pH. To avoid irritation of the pulmonary mucosa, pH ranges between 5 and 8 should be utilized for nebulized products, however, a pH as low as 3.5 may be acceptable. For tolerability, an isotonic formulation should be targeted. Various buffer solutions have been evaluated for their effect on mAb aggregation after nebulization. In combination with PS80, acetate and histadine, buffered solutions were shown to have a lower occurrence of aggregation in comparison to citrate-buffered solutions [24]. The mechanism by which these buffers affect aggregation is complex, dependent on pH range and sensitive to fluctuations in temperature as well as protein isoelectric point and diffusion coefficient.

Chemically sensitive biologics, such as nucleic acids, have been reported for treatment of many genetic diseases [25]. Nucleic acids, in general, are unstable and degrade quite easily in physiological fluids [26]. The most common form of degradation is hydrolysis, due to the affinity of the reaction of water with labile phosphodiester linkages [27]. Nucleic acids can be protected from hydrolysis by encapsulation within a lipid membrane. This approach was used to produce COVID-19 vaccines, where a mRNA strand is encapsulated within a lipid nanoparticle structure. The structure of LNP's contain four major lipid components: helper lipid, cholesterol, PEGylated lipid and ionizable lipid. Generally, lipids have structural characteristics, such that one part of the molecule is hydrophilic while the other is hydrophobic. Each of these components plays a significant role in the stability, geometry and function of the LNP system [28]. For instance, the nature of the ionizable lipid will dictate whether the electrostatic encapsulation of the nucleic acid is strong enough to retain it in the aqueous cavity of the LNP. In terms of the outer shell of the LNP, a PEGylated lipid provides solubility and stealth *in vivo*, such that the LNP presents a longer half-life while avoiding phagocytosis [29].

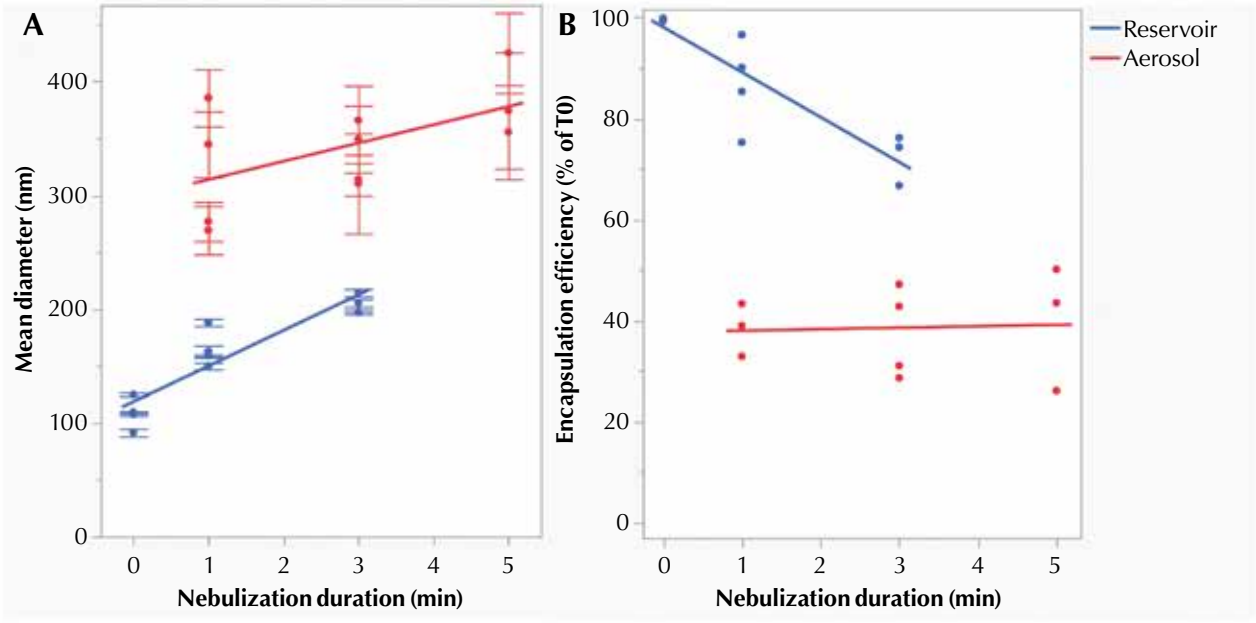
Typically, LNP's are formulated for subcutaneous or intramuscular injections for treatment of various diseases, however recently, these formulations have been altered to support aerosolization for administration to the lung [29, 30]. In the case of stability concerns, namely LNP aggregation and/or agglomeration, viscosity and dispersibility enhancers are added such that distance between LNPs is maximized [28, 31]. Various commercial air jet and vibrating mesh nebulizers have been utilized to determine the effect of nebulization on LNP and cargo integrity [32].

Case study: The effect of nebulization stress on LNP stability

Biologic stability during nebulization is a key indicator of potential performance during nebulization. Multiple stability assessments must be considered to determine the chemical and physical effects on biologic integrity. Stresses can include exposure to the

Figure 4

Time-dependent LNP instability during vibrating mesh nebulization. LNP size (a) and encapsulation (b) over the nebulization duration in the reservoir (blue) and collected aerosol (red).



liquid-air interface, concentration due to solvent evaporation and local heating from the nebulization process. Such stresses can cause protein degradation (oxidation) and aggregation, which in turn, can cause denaturation [33]. Other forms of biologics like nucleic acids are typically encapsulated within LNP's before nebulization in a suitable vehicle. The stresses described here also apply to these particle systems and can affect not only particle stability, but also cargo stability.

To test these effects, we formulated polyA-loaded LNP's. The geometric diameter of these LNP's as determined by dynamic light scattering was 80-100 nm, depending on the lipid formulation components and mol ratios. The vehicle was developed to contain excipients that will allow LNP's to become dispersed and suspended within the nebulized droplet, such as viscosity modifiers and various surfactants. To determine the impact of nebulization stresses on the formulation, we utilized off-the-shelf nebulizers. In the study, we wanted not only to determine the effect on LNP particle size but also on polyA encapsulation. These LNP size and aerosolized encapsulation efficiencies were assessed at two points in the nebulization process. We assessed the effect on the formulation inside the medication chamber, which experiences some of the nebulizer energy without becoming aerosolized, as well as liquid collected post-nebulization.

The aerosolized formulation was collected in a cold tube to support condensation and maximize collection efficiencies. Methodologies for collection of aerosols have been studied and utilized over a wide range of applications, such as air sampling and nebulized aerosol collections. These collection methods are mostly categorized as impingers, cyclones, impactors and filters [34]. Impingers like the BioSampler® (SKC Inc., Eighty Four, PA, US) and cyclones draw aerosol through a vacuum, which then collects in a liquid medium. Impactors like the BioStage® (SKC Inc., Eighty Four, PA, US) or Anderson Cascade Impactor and filters utilize a fine membrane to trap aerosols. Each technique could induce structural perturbations of the lipid nanoparticles in addition to variable collection efficiencies and would require initial screening to select the optimal collection method.

The lipid nanoparticle diameter was assessed pre- and post-nebulization using dynamic light scattering. The pre- and post-encapsulation efficiency was measured using a ribogreen assay. Results are summarized in Figure 4, where mean diameter and encapsulation efficiency are plotted as a function of nebulization duration.

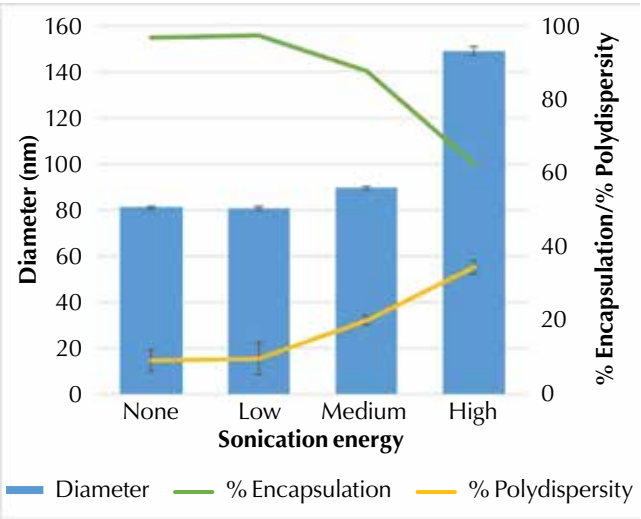
As Figure 4 shows, there is an effect on both diameter and encapsulation efficiency of LNPs as vibrating mesh nebulization time increases. Notably, the diameter of the LNPs that reside in the reservoir are affected by vibrational energy, causing destabilization of nominal particle size and encapsulation efficiency. Post-nebulized LNPs demonstrate a greater effect from the stresses of nebulization, as evident in a size increase from ~ 100 nm to > 400 nm, indicating either aggregation of individual LNPs or agglomeration via coalescence into larger single particles. Due to the small size of the lipid nanoparticles, their high surface energies can lead to aggregation (clustering) to minimize those energies. Alternatively, the process of nebulization could cause the spherical particles to become disk-like, which increases the surface area for dispersion forces to become more prevalent between particles [35, 36]. Interestingly, the nebulization effects seem to reveal a leakage effect of the cargo polyA. It is possible that during nebulization, the intraparticular structure is perturbed, such that polyA leaks (diffuses) through the lipid bilayer. As nebulization increases, significantly more cargo is leaked into the surrounding medium, indicating either a rupture event in the lipid membrane or simple diffusion during membrane merging.

To further assess whether faster screening can be developed to determine the impact of vibrational stress on polyA-loaded LNPs, we utilized lab sonication as a comparable ultrasound energy input. Sonication at 35 kHz was applied under increasing modes of energy output (none, low, medium and high) on the polyA-loaded LNPs, as summarized in Figure 5.

Figure 5 demonstrates that as sonication energy increases over time, LNP diameter and polydispersity increase and encapsulation efficiency decreases. Specifically, in the presence of "high" sonication energy, the LNP particle size increase to ~ 140 nm and the encapsulation efficiency decreases to ~ 60%, which indicates major leakage of polyA. However, relatively "low" sonication energy preserves the structural integrity and encapuslation efficiency. This result

Figure 5

The effect of externally applied sonication energy on LNP mean diameter, polydispersity (%) and encapsulation (%).



suggests that lower sonication energy preserves LNP integrity not only in particle size but encapsulation efficiency. These results simply suggest that exposing lower nebulization energy to the LNP formulation could lead to unperturbed aerosolizable LNPs.

Using a lab sonicator, a prediction may be made to determine the impact of nebulization vibrational stress on LNP stability: If the amount of energy input (J/g) is determined for the specific nebulizer to facilitate the nebulization process, then by simple dimensional analysis, the time equivalence can be determined from the parameters of the bath sonicator (powder per mass of medium (J/(g · s)). This same concept could be applied to other biologics, where these energy stresses could be minimized. The sonication method itself is a useful tool; however, further comparable energies could be taken into account, such the energy transfer process from sonic energy through vialed liquid.

Overall, the ability to minimize stresses on aerosolized formulations of biologics is of utmost importance. Key steps include formulation composition, where excipients such as buffers and salts, as well as viscosity and tonicity modifiers, and suspension enhancers can minimize stresses.

Conclusion

Increasingly, inhaled biotherapeutics are being utilized to treat a wide array of diseases, leveraging the unique advantages of pulmonary delivery. However, delivering biologics to the lungs presents challenges related to maintaining stability and potency during nebulization. The choice of nebulization device—whether air jet, ultrasonic, vibrating mesh or soft mist—greatly influences the preservation of biologic integrity, with each technology offering distinct benefits and limitations. Formulation strategies, including the use of surfactants, stabilizers and optimized buffers, are crucial for mitigating degradation during aerosolization. Evaluating nebulization of lipid nanoparticle-encapsulated nucleic acids underscores the need for continued innovation in both formulation and delivery technologies. Moving forward, refining these approaches will be essential to fully realize the potential of inhaled biotherapeutics for a broader range of conditions.

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Matthew J. Owen, PhD, is Group Leader, Product Development; David Wilcox, BS, is Director, Inhalation and Alan B. Watts, PhD, is Director of Innovation and Partnerships for Orally Inhaled Products at Catalent Pharma Solutions, 14 School House Road, Somerset, NJ, 08873, US. Corresponding author: Alan B. Watts, PhD, alan.watts1@catalent.com, www.catalent.com.