Formulation, Device, and Clinical Factors Influencing the Targeted Delivery of COVID-19 Vaccines to the Lungs

Sayeed Mossadeq[1] · **Rajen Shah**[1] · **Viraj Shah**[1] · **Milind Bagul**[1]

Abstract

The COVID-19 pandemic has proven to be an unprecedented health crisis in the human history with more than 5 million deaths worldwide caused to the SARS-CoV-2 and its variants (https://www.who.int/emergencies/diseases/novel-coronavirus-2019). The currently authorized lipid nanoparticle (LNP)–encapsulated mRNA vaccines have been shown to have more than 90% vaccine efficacy at preventing COVID-19 illness (Baden *et al*. New England J Med 384(5):403–416, 2021; Thomas *et al*., 2021). In addition to vaccines, other small molecules belonging to the class of anti-viral and anti-inflammatory compounds have also been prescribed to reduce the viral proliferation and the associated cytokine storm. These anti-viral and anti-inflammatory compounds have also been shown to be effective in reducing COVID-19 exacerbations especially in reducing the host inflammatory response to SARS-CoV-2. However, all of the currently FDA-authorized vaccines for COVID-19 are meant for intramuscular injection directly into the systemic circulation. Also, most of the small molecules investigated for their anti-COVID-19 efficacy have also been explored using the intravenous route with a few of them explored for the inhalation route (Ramakrishnan *et al*. Lancet Respir Med 9:763–772, 2021; Horby *et al*. N Engl J Med 384(8):693–704, 2021). The fact that the SARS-CoV-2 enters the human body mainly via the nasal and airway route resulting in the lungs being the primary organs of infection as characterized by acute respiratory distress syndrome (ARDS)–mediated cytokine storm in the alveolar region has made the inhalation route gain significant attention for the purposes of targeting both vaccines and small molecules to the lungs (Mitchell *et al*., J Aerosol Med Pulm Drug Deliv 33(4):235–8, 2020). While there have been many studies reporting the safety and efficacy of targeting various therapeutics to the lungs to treat COVID-19, there is still a need to match the choice of inhalation formulation and the delivery device platform itself with the patient-related factors like breathing pattern and respiratory rate as seen in a clinical setting. In that perspective, this review aims to describe the various formulation and patient-related clinical factors that can play an important role in the judicious choice of the inhalation delivery platforms or devices for the development of inhaled COVID-19 vaccines.

Keywords ARDS · dry powder inhalers · inhalation · inhaled COVID-19 vaccines · mRNA vaccine · mucosal immunity · nebulizers · pharmaceutical inhalers · pressurized metered dose inhalers · SARS-CoV-2 · targeted vaccine delivery

Introduction

The SARS-CoV-2 that caused pandemic COVID-19 is a highly contagious life-threatening infectious disease that has resulted in the death of more than 5 million people worldwide within a span of 2 years of its outbreak [1]. The virus has been primarily found to infiltrate into the human biological system via the nasal and airway route as a result of inhalation of airborne aerosol droplets that can get seeded in the mucosa of the nasal and airway epithelium. Following seeding of the virus particles in the nasal mucosa, local inflammatory reactions to the various viral antigens (nucleocapsid protein, membrane protein, envelope, and spike protein) can follow leading to excessive mucus production.

The excessive mucus produced can further be subject to inhalation via the nasal route causing the aerosolization of the mucus containing virus particles within the nasal

✉ Sayeed Mossadeq
Sayeed.mossadeq@raptimresearch.com;
Sayeed2222@gmail.com

1   Raptim Research Private Limited, 1378 Rt.206., STE 6/280, Skillman, NJ 08558, USA

cavity at the opening aperture of the naso-pharynx, which can then be inhaled into the upper and peripheral airways, thus leading to further seeding of different lung regions with the virus particles [2, 3]. Thus, it is important to consider the involvement of upper airway mucosal surface and the nasal mucosa in the initial stages of the infectious disease in order to develop successful treatment strategies that can trigger mucosal immunity at the primary sites of viral infiltration. Such a strategy of targeting vaccines to the nasal and airway mucosa might prove to be better in limiting and preventing the viral proliferation and inflammation from progressing further and thus could result in better clinical outcomes compared to traditional intravenous and intramuscular routes of vaccine delivery. Furthermore, the virus has also been reported to gain entry into the host biological system mainly via the angiotensin-converting enzyme (ACE2) receptors that are highly expressed throughout the upper airways—thus making the airways highly susceptible to SARS-CoV-2 infection [4–9]. The very high expression of ACE2 on the surface of the human upper airways and also the nasal epithelium thus makes the targeted delivery of vaccines and therapeutics to the lungs a better treatment strategy that could achieve a better safety-efficacy profile compared to traditional injection routes.

Furthermore, the major cause of death in COVID-19 patients infected with the virus has been reported to be triggered mainly by acute respiratory distress syndrome (ARDS) in the alveolar region of the lungs wherein the excessive cytokine storm caused by the viral infiltration into alveolar macrophages leads to a cascade of pathological events. These pathological events in the alveolar region triggered by the cytokine storm can range from loss of alveolar epithelium integrity due to the damaging effect of the cytokines on the alveolar epithelium leading to interstitial fluid infiltration into the alveolar space, to the loss of pulmonary alveolar surfactant integrity as a result of interstitial fluid infiltration into alveolar space. The fluid collected in the alveolar space is believed to be capable of causing impaired oxygenation of the blood at the air-blood interface leading to suffocation and death of infected patients via ARDS. Thus, the ARDS seen in COVID-19 patients is a result of a combination of pathological conditions causing diffuse alveolar damage consisting of permanent damage to alveolar epithelial cells and capillary endothelial cells [10, 11].

Therefore, there is ample evidence highly acknowledged by the scientific community that the major route of SARS-CoV-2 and its variants' infiltration into the human biological system is via the oral inhalation and nasal route with the lungs being the primary organ of destruction. Thus, the targeted delivery of vaccines and therapeutics to the lungs via the oral inhalation route and nasal route can be expected to have a better safety-efficacy profile compared to intramuscular and intravenous routes for treating COVID-19

complications [12,13]. However, all the currently FDA-authorized COVID-19 vaccines are intended for delivery to the human body via the intramuscular route, specifically the mRNA-1273 and BNT162b2 (COMIRNATY) vaccines from Moderna and Pfizer respectively [14, 15]. These intramuscularly administered vaccines mainly function by initiating systemic immunity primarily more than mucosal immunity that can lead to increased levels of serum IgG antibodies in the systemic circulation but lower levels of IgA antibodies. Such high levels of systemic immunity characterized by higher levels of serum IgG antibodies but relatively lower levels of mucosal IgA antibodies following intramuscular administration of both the first and second doses of both the Moderna (mRNA-1273) and Pfizer (BNT162b2) vaccines have been reported by many authors [16, 17].

Furthermore, it has been reported by Julian *et al*. (2021) that both the mRNA vaccines that are currently FDA authorized (mRNA-1273 and BNT162b2) resulted in high levels of IgG and relatively lower levels of IgA in serum following administration of both the first and second doses of the vaccines [18]. However, the authors reported that for both the mRNA vaccines, only the serum IgG levels remained elevated during the 21-day follow-up period post first vaccination and also remained elevated during the 20–50-day follow-up period post second dose. The serum IgA levels were reported to decline rapidly following the intramuscular administration of both the first and second doses of both the mRNA vaccines, thus highlighting the possible inability of intramuscular route of mRNA vaccine administration to maintain the IgA levels at an elevated level for a longer time.

To give a brief introduction about antibodies, immunoglobulins (Ig) that are secreted by specialized cells of the adaptive immune system are basically heterodimeric proteins composed of 2 heavy and 2 light chains of polypeptides [19]. The Igs contain a variable domain that binds to antigens and a constant domain for the recognition and binding to effector cells. Based on the differences in the constant domain region, the Igs can be classified into five types, namely IgG, IgA, IgM, IgD, and IgE. Based on the differences in the variable domains, the specificity of the antibodies to viral antigens is dictated. Human IgG is further classified into IgG1, IgG2, IgG3, IgG4, and IgG5 based on the differences in their structure related to the location and number of inter-chain disulfide bonds. Human IgA exists as two subtypes IgA1 and IgA2 based on the structural difference in the hinge region present between the two Fab arms and the Fc region. The IgA1 is found to have an extended hinge (absent in IgA2) that confers on its higher avidity towards distantly located antigens while at the same time increasing its vulnerability to enzymatic degradation. The serum IgA which is mainly synthesized in the bone marrow exists predominantly of the monomeric IgA1 type, whereas the mucosal IgA which is synthesized in mucosal

lymphoid cells (in lamina propria) exists both as IgA1 and IgA2 in mostly the dimeric form [20]. The locally produced IgA is transported by the polymeric immunoglobulin receptor (PIGR) through the epithelial cells and released at the mucosal surface via the cleaving of the PIGR. Mucosal transport of the IgG happens via pinocytosis and/or FcRn receptor–mediated uptake within acidified vacuoles that are then apically expressed via transcytosis.

In that perspective, it is important to note the findings of Sterlin *et al*. (2021) who have reported that the IgA antibodies are more effective in neutralizing the SARS-CoV-2 compared to IgG. The authors have shown that although the abundance of IgG is about 5 times compared to that of IgA, the IC50 of IgA was found to be about 7 times lower than that of IgG for the neutralizing potential against SARS-CoV-2 [21]. Furthermore, the authors have also reported that the IgA levels against the viral receptor binding domain (RBD) were higher both in the broncho-alveolar lavage (BAL) and saliva compared to anti-RBD serum and mucosal IgG levels implying that IgA might be offering higher degree of protection against COVID-19 in both serum and at the mucosal surface. Thus, there is significant evidence to show that the antibodies IgA have greater potential to limit the spread and pathogenesis of COVID-19 compared to serum IgG. The authors have also shown that the anti-RBD IgA levels at the mucosal surface were persistently higher compared to their peripheral blood levels in hospitalized COVID-19 patients [21].

The reason for such observed superiority of IgA over IgG in neutralizing SARS-CoV-2 is attributed to the increased flexibility and longer hinge structure among many other more favorable structures of IgA compared to IgG that enables IgA to interact with improved access to SARS-CoV-2 trimer [21, 22]. The other reasons like higher cross-reactivity of IgA against various corona viruses compared to IgG and the delay in maturation of systemic IgG compared to mucosal IgA have also been cited for the higher efficacy of mucosal IgA against the virus [23]. The dimeric and/or polymeric nature of mucosal IgA has also been suggested to enable better cross-reactivity with SARS-CoV-2 spike proteins compared to monomeric forms of IgG 24. Thus, the targeted delivery of COVID-19 vaccines to the mucosal tissue might be more beneficial in offering protection against the virus compared to other routes of administration. The higher immunity response derivable from inhaled route could be via triggering of mucosal immunity compared to IgG only–based protection derivable via intramuscular injection. Such better protective effects for the inhaled route of vaccine administration over other routes have also been reported in animal studies 25.

Accordingly, many organizations have investigated the targeted delivery of their vaccine candidates to the mucosal surface of the nasal and airway epithelium with an aim to trigger mucosal immunity to offer protection against COVID-19. The first of the organizations to investigate the inhaled route for the targeted delivery of vaccines to treat COVID-19 was the Imperial College in London, who with their Landmark trial aimed to develop an inhaled version of their siRNA vaccine [26]. Following that there have been many corporate and academic organizations that have ventured into the development of inhaled and nasal spray versions of COVID-19 vaccines with an aim to trigger the mucosal immunity to combat COVID-19. While most of these organizations in the inhaled COVID-19 vaccine space have explored and are currently in the process of developing aqueous formulation-based COVID-19 vaccines, some of them are also exploring dry powder versions of the vaccine. While the detailed list of organizations active in the development of inhaled and nasal versions of the COVID-19 vaccines is not the primary objective of this review, the readers are directed to another review enlisting these organizations [27].

Apart from vaccines, the targeted delivery of small molecules especially already FDA-approved anti-viral and anti-inflammatory compounds have also been investigated for their efficacy in treating COVID-19 [28]. For a detailed list of inhaled anti-viral and anti-inflammatory compounds that have been investigated for COVID-19 treatment, the reader is directed to a review by Eedara *et al*. [12]. Inhaled dry powder of budesonide was shown to be successful in preventing exacerbation of COVID-19 symptoms when administered early in the disease onset in the STOIC clinical trial by Ramakrishnan *et al*. [29]. Similar positive results were also reported for inhaled budesonide dry powder version by Yu *et al*. 30 wherein the authors have reported that the dry powder version of the steroid improved the time to recovery while reducing hospital admissions in COVID-19 patients who are at risks of complications. Likewise, a positive benefit for COVID-19 patients was also reported for inhaled ciclesonide pressurized metered dose inhaler (pMDI) by Iwabuchi *et al*. [31]; however, Clemency *et al*. [32] have reported that the ciclesonide pMDI administration did not achieve the primary efficacy end point of reduced time for alleviation of COVID-19 symptoms in a randomized clinical trial. Such differences in treatment success could be attributed to the patient-related factors like respiratory rate that could have a vital impact on the deposition of inhaled therapeutics in the lungs when coupled with the specific breathing pattern requirement for every inhaler. This is because the breathing pattern and inhalation flow rates presented by COVID-19 patients have an important effect on the air flow dynamics within the lungs which in turn can dictate the major mechanisms of aerosol deposition within the different lung regions. Thus, in addition to targeted delivery of COVID-19 vaccines to the lungs, the choice of the

specific type of inhaler can also have an important effect on the successful immunity development.

The breathing pattern and inhalation flow rates presented by COVID-19 patients is known to vary significantly from that of healthy people. This is mainly because of the fluid infiltration into the alveolar region of the lungs leading to acute respiratory distress syndrome (ARDS), wherein the patients develop rapid and shallow breathing profiles with significantly increased respiratory rates. Typically in COVID-19 patients with ARDS, respiratory rates more than the normal rate of 16 breathes per minute are recorded which can be in the range of 24–30 breathes per minute [33] and in some extreme cases respiratory rates of more than 30 have also been reported [34]. Thus, the diffuse alveolar damage seen in COVID-19 patients combined with the fluid infiltration into the alveolar space results in serious breathing difficulties for the COVID-19 patients.

Apart from the alveolar region damage–mediated ARDS, the virus has also been reported to enter into the brain tissue via the olfactory bulb mainly and accumulate in the respiratory center of the brain, especially in the brainstem. The viral infiltration into the respiratory center in the brainstem could thus lead to further impairment of normal breathing patterns observed in COVID-19 patients. Dey *et al*. [35] have reviewed the various physiological routes via which the virus can infiltrate into the brain and ultimately end up accumulating in the brainstem [35]. The authors have discussed several hypothetical routes for the viral entry into the brainstem including viral entry into the brainstem from the systemic circulation as a result of the blood–brain barrier compromise. The virus has also been thought to gain access into the brain from the lungs via the vagus nerve and pulmonary stretch receptor routes. Thus, there are multiple physiological routes the virus can utilize in order to infiltrate into the brain, leading to the impairment of the normal brainstem functioning paving the way to abnormal breathing patterns in COVID-19 patients.

Satturwar *et al*. [36] have also reported post-mortem evidences of SARS-CoV-2 infiltration into the brain of patients who died from COVID-19 including characteristics like hemorrhage, acute infarction, and thrombi in the brains as indicated by their autopsy report. Similar post-mortem reports proving the presence of viral load in the brainstem and olfactory bulb of patients who died from COVID-19 have been reported by Menter *et al*. [37]. Meinhardt *et al*. [38] have also reported significant copies of the viral RNA in the respiratory center of the brain in addition to many other organs. Therefore, there is significant clinical evidence to show that the virus can indeed infiltrate via many routes into different regions of the human brain and can also preferentially accumulate within the respiratory centers of the brain that control important involuntary processes of breathing in humans.

Given the proven viral infiltration into the respiratory center in the brain combined with the ARDS symptoms seen in COVID-19 patients, a direct implication of such a clinical manifestation would be the difficulty for COVID-19 patients to inhale appropriately or sufficiently from any pharmaceutical inhaler. Furthermore, the differences in COVID-19 progression in patients and the degree of illness can also add to the variability in observable breathing patterns in COVID-19 patients. Thus, it is very important to include the clinical factor of abnormal breathing pattern differences in COVID-19 patient population while choosing an appropriate inhalation device for the targeted delivery of vaccines and therapeutics to treat COVID-19. This is because the lung deposition of inhaled therapeutics and vaccines is significantly affected by the breathing pattern and inhalation maneuver presented by the patients during inspiration from the inhaler [39]. Also, there are different types of pharmaceutical inhalers like pressurized metered dose inhalers (pMDIs), dry powder inhalers (DPIs), and nebulizers in the market that require different and specific types of breathing patterns for their correct operation in order to achieve optimal lung deposition of inhaled therapeutics using those inhalers [40, 41].

Furthermore, it is commonly observed in a clinical setting that for any given breathing pattern, the lung deposition and emitted dose can vary between different types of inhalers used for the delivery of the same therapeutics. Melchor *et al*. [42] have reported that administration of same dose of 200 μg of salbutamol from a pMDI resulted in nominally higher peripheral lung deposition of salbutamol of 44.1% compared to 39.4% for the DPI, whereas the use of a spacer with the pMDI resulted in significantly higher salbutamol dose deposition of 49.4% in the peripheral lung compared to the DPI. Thus, the study by Melchor *et al*. [42] done in normal subjects and asthmatics clearly showed the difference in lung deliverable dose achievable for the same API at similar doses when delivered using different inhaler types. Therefore, the rapid and shallow breathing patterns combined with the variability in the degree of breathing difficulties as a function of disease intensity typically seen in COVID-19 patients may not permit the prescription or usage of one universal inhaler type for all COVID-19 patient population. Such a strong influence of breathing patterns on the regional lung deposition of inhaled dose of any small or large molecule requires the optimization and correlation of the formulation factors with that of the clinical factors seen in the COVID-19 patient population of interest for whom the targeted delivery of COVID-19 therapeutics and vaccines would be developed.

Apart from patient inhalation patterns, the other factors that are known to affect the regional lung deposition of inhaled therapeutics are the formulation factors and device design factors that can modulate the aerosolization efficiency via their effect on the magnitude of aerodynamic

shear forces responsible for the breakage of the bulk formulation into smaller aerosol droplets. These formulation factors can also vary between the different types of inhalers in use like, for example, in the case of aqueous nebulizer formulations, surface tension and viscosity could be considered few of the major parameters that can affect the primary aerosol droplet diameter and regional lung deposition. In the case of the pMDIs, the vapor pressure of the formulation which is mainly a function of hydrofluoroalkane (HFA) propellant and co-solvent concentration can have a major influence on drug deposition in lungs [43, 44]. For the DPIs, the intra-particle cohesive and inter-particle adhesive forces of attraction in the active pharmaceutical ingredient (API)-lactose carrier blend are major formulation factors influencing the deposition of inhaled therapeutics in the lungs [45]. This is because in the case of DPIs, the inspiratory force generated by the patient's inhalation pattern from the DPI is required for the de-aggregation of the API from the lactose carrier.

The various device design factors that can play an important role in the emitted dose and lung deliverable dose can also be categorized based on the specific type of inhaler in use. For example, in the case of pMDIs, the device design factors like spray orifice diameter, land length, volumes of metering, and expansion chamber are some of the major device parameters that have a profound influence on the aerosol droplet diameter distribution, spray pattern, plume geometry, and the overall performance of the pMDIs. Duke *et al*. [46] have reported that decreasing the spray orifice diameter has the effect of increasing both the plume velocity and fine particle fraction keeping the vapor pressure of the pMDI formulation constant [46]. In the case of DPIs, one of the major device parameters that can affect the fine particle fraction is the internal mesh design that is primarily responsible for the device resistance that needs to be overcome by the patient's inhalation flow rate in order to enable optimal de-aggregation of the API drug particles from the lactose carrier. Generally, it is considered that DPI devices with high resistance could result in higher fine particle fractions as a result of better de-aggregation of APIs from the lactose carrier, compared to DPIs with lower resistances [47]. Such a trend in fine particle fraction *versus* DPI resistance is attributable to the effective conversion of the kinetic energy from the patient's inspiratory flow rate to aerodynamic shear forces that ultimately dictate the magnitude of API de-aggregation from the lactose carrier. In the case of nebulizers, one of the major device-related parameters that can be cited to affect the aerosol droplet diameter and thus the deliverable dose to the lungs is the vibration frequency of the piezoelectric crystal (in the case of ultrasonic nebulizers) or the air-jet flow velocity (in the case of air-jet nebulizers) that dictate the mechanism of aerosolization in nebulizers [48, 49].

Thus, there are multiple factors including mainly the formulation, device-related, patient-related, and clinical factors that can influence the targeted delivery of COVID-19 therapeutics and vaccines to the lungs. These factors not only are capable of influencing the lung deliverable dose and other major performance parameters in an independent manner but they can also exert their influence on the treatment success in a complex intertwined manner wherein these factors can cross-influence each other as well. In that regard, this review aims to discuss the various formulation, device, and clinical factors that need to be taken into consideration during the development of inhaled COVID-19 vaccines to the lungs. This review will focus specifically on the orally inhaled COVID-19 vaccine development strategies taking the example of the recently FDA-authorized mRNA vaccines from Moderna and Pfizer. This review aims to discuss the various formulation, device, and clinical strategies that are applicable towards the development of an orally inhaled version of the currently FDA-approved mRNA vaccines described in the perspective of the complex interplay between device factors and patient-related clinical factors. The review makes a note of manufacturer information for certain equipment and devices, only for the purposes of highlighting the relevant information for the quick reference of the reader and does not intend to promote any specific brand in any way.

The review expects the reader to visualize a possible perspective of the concept of vaccination in parallel to its commonly held prophylactic perspective. Such a parallel perspective on vaccination is to be visualized in light of the highly mutative nature of SARS-CoV-2 that could enable the virus to evade specific type of vaccination in an obscure manner. Thus, an individual vaccinated with one specific type of vaccine against a specific strain of the virus might still carry the risk of being infected by mutant strains immune to the administered vaccine. In that perspective, the higher levels of mucosal antibodies the COVID-19 patients and/or healthy individuals can generate within their body via inhaling the mRNA vaccine compared to other routes of administration could increase their chances of being protected from more virulent strains yet to infect them. Thus, the common perspective of vaccine administration applicable only for healthy individuals is to be critically visualized in the light of the highly transient nature of SARS-CoV-2 and its variants.

Description of Moderna's mRNA COVID-19 Vaccine Formulation mRNA-1273

Each 0.5 ml of the Moderna mRNA-1273 vaccine contains 100 μg of nucleoside-modified messenger RNA (mRNA – single stranded 5′-capped) encoding the pre-fusion stabilized spike glycoprotein (S) of the SARS-CoV-2 virus

[50–53]. The vaccine is supplied as a sterile white to off-white frozen suspension (to be thawed prior to use to a liquid phase) for intramuscular (IM) injection in total bulk volumes of 5.5 ml and 7.5 ml in the vial. The mRNA is formulated or encapsulated within lipid nanoparticles (LNPs) of average diameter typically varying between 60 and 100 nm [54] as characterized by dynamic light scattering (DLS). The different types of lipids that make up the LNP in the mRNA-1273 vaccine are the proprietary ionizable lipid SM-102 which is a synthetic amino lipid, polyethylene glycol (PEG 2000)—dimyristoyl glycerol (DMG), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), and cholesterol in the molar ratio of 50:10:38.5:1.5 making up a total lipid content of 1.93 mg.

The encapsulation of the mRNA within the LNPs enables not only the protection of the naked mRNA from various extracellular host degradative factors but also plays an important role in the uptake of the mRNA by the host cell. The encapsulation efficiency of the mRNA within the LNPs is however not revealed in any of the documents which could give an estimate of the percentage of mRNA present in the extracellular space compared to the encapsulated mass. The other inactive ingredients present in the mRNA-1273 COVID-19 vaccine suspension are 0.31 mg tromethamine, 1.18 mg tromethamine hydrochloloride, 0.043 mg acetic acid, 0.2 mg sodium acetate trihydrate, and 43.5 mg sucrose, water for injection. The tromethamine entities (Tris) along with acetic acid are used as buffers to maintain the pH of the formulation at 7–8 (precisely 7.5), while the sucrose serves as a medium of protection from extreme cold temperatures of storage required for maintaining the stability of the vaccine. Thus, it should be noted that out of the 0.5 ml of the total bulk aqueous–based formulation, about 47.263 mg is composed of chemical entities other than water, making the total weight percentage of water in the formulation to be around 91% w/w. The storage conditions for the mRNA-1273 are specified at − 50 to − 15°C for newly received vials and after first dose has been withdrawn the vials can be stored at 2–25°C for up to 6 h [51–53].

Description of Pfizer's mRNA COVID-19 Vaccine Formulation BNT162b2 (COMIRNATY)

Each 0.3 ml dose of the Pfizer BioNTech BNT162b2 vaccine (now branded as COMIRNATY) contains 30 µg of nucleoside-modified messenger RNA (mRNA single stranded 5′-capped) encoding the spike glycoprotein (S) of the SARS-CoV-2 virus [55–58]. The vaccine is supplied as a sterile frozen suspension (− 80 to − 60°C), which is to be thawed prior to use to a liquid phase using sterile 1.8 ml of sterile 0.9% NaCl (supplied separately), for intramuscular (IM) injection in total multi-dose bulk volumes of 0.45 ml, 2 ml,

and 5 ml in the vial. The mRNA is formulated or encapsulated within lipid nanoparticles (LNPs) of average diameter typically varying between 60 and 100 nanometers [54] as characterized by dynamic light scattering (DLS). The different types of lipids that make up the LNP in the mRNA-1273 vaccine are the ionizable cationic lipid ALC-0315, ALC-0159, 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), and cholesterol in the molar ratio of 46.3:9.4:42.7:1.6 making up a total lipid content of 0.77 mg [54].

The other inactive ingredients in the vaccine suspension are 0.01 mg potassium di-hydrogen phosphate, 0.07 mg disodium hydrogen phosphate dehydrate pH 7–8, 0.01 mg potassium chloride, 0.36 mg sodium chloride, and 6 mg sucrose in water for injection (WFI). The vials are labeled to be stored at ultra-low temperatures of − 90 to − 60°C until expiry date printed on label or they can also be stored at − 25 to − 15°C for up to 2 weeks. For more details on the storage conditions of the vaccine, the reader is referred to references 48–51. Thus, overall it can be seen that both the mRNA-1273 and the COMIRNATY vaccines contain specific masses of viral spike protein mRNA encapsulated within LNPs comprising of different types of lipids. Therefore, the overall structural makeup of both the mRNA vaccines can be thought to be analogous to each other in that both vaccines have mechanisms of intended uptake by the host cells via LNP-mediated host-cell interactions. This commonality of mRNA-LNP encapsulation and LNP host-cell interaction–driven vaccine uptake mechanisms is cited on the grounds of their importance for the development of inhaled formulation versions of the mRNA-LNP vaccines. The following sections would describe the various inhalation platforms that can be explored for the development of inhaled versions of the mRNA-1273 and COMIRNATY vaccines.

Formulation, Device, and Patient-Related Clinical Factors to Consider in the Development of pMDI Versions of the COVID-19 mRNA Vaccines

Pressurized metered dose inhalers (pMDIs) are multi-dose inhalers that typically contain the active pharmaceutical ingredient (API) either dissolved or suspended in almost completely liquefied high vapor pressure propellants contained within metallic canisters [59, 60]. The most commonly used propellants in the currently marketed pMDIs are the hydrofluoroalkane (HFA) propellants that replaced the chlorofluorocarbons as a result of the Montreal Protocol [61, 62]. HFA-134a and HFA-227 are the most commonly used HFA propellants that can either be used alone or in combination with each other in different molar ratios depending on the vapor pressure required for aerosolization of the pMDI formulation. Although recently the HFA-152a from Koura [63] is in the development phase promising to prove as better environment-friendly pMDI dispersion matrix compared to

other HFA propellants, there are currently no FDA-approved or -marketed pMDIs using HFA-152a. Apart from the HFA propellants, other excipients like ethanol and surfactants can also be incorporated into the formulation with an aim to improve the solubility of the API(s) and/or to modify the vapor pressure of the formulation. The optimization of pMDI formulation parameters like API solubility and vapor pressure via excipients can be useful to improve dosing reproducibility and prevent undesired API agglomeration.

The pMDIs frequently have held the largest market share of all the pharmaceutical inhaler types sold in the USA which was estimated at about 75% (55.3 million units of pMDI sold in 2019) of the total market share of inhalers compared to only 25% (18.9 million units of DPIs sold in 2019) contributed by dry powder inhalers (DPIs) [64]. A similar trend in the increased sales of pMDIs (about 70%) over DPIs and nebulizers has also been reported for European countries by Janson *et al*. (2019) and Lavorini *et al*. (2011) [65, 66]. Furthermore, the pMDIs are also a popular choice among COPD and asthma patients because of their portability, disposability, and relatively lower cost [65, 67]. In spite of their extensive market share and being the most popular choice of inhalation device among patients and clinicians, the pMDIs have not been extensively investigated for their suitability to deliver a wide variety of macromolecules like peptides, proteins, nucleic acids, and other biologics compared to DPIs and nebulizers.

The APIs in the pMDI-HFA-excipient formulation are held at high pressures of more than 3–5 bars within metallic canisters (typically made of aluminum alloys and in some cases stainless steel) due to the sealing of the metal canister with the metering valve via a mechanical crimping process. The pMDI canisters are supplied with a mouth-piece actuator typically made of inert plastic materials with built-in laser drilled spray orifice and intricate micrometer scale channels for the passage of the pMDIs internal high-pressure formulation to the external atmosphere. The mouth-piece actuators mainly serve to provide the necessary aerodynamic shear forces for the breakage of the metered pMDI formulation into micron-sized smaller aerosol droplets [68]. The mechanism of aerosol formation in pMDIs is based on the high vapor pressure of the HFA propellants enabling the ultra-rapid flash evaporation of the liquid portion of the propellant to vapor phase which is initiated within the expansion chamber of the metering valve upon depression of the pMDI canister. This ultra-rapid flash evaporation of the pMDI formulation proceeds all through the land length (the hollow cylindrical passage connecting the actuator's expansion chamber with its spray orifice) and spray orifice of the mouth-piece actuator producing a fine spray comprised of aerosol droplets containing the API(s) which can then be inhaled by the patient. The primary aerosol droplet diameter values at the mouth-piece exit are expected to be in the range

of 1–5 microns and generally considered to be suitable for inhalation [69].

Therefore, to formulate the COVID-19 mRNA-LNP vaccines in a pMDI-HFA format, the physiochemical compatibility of the mRNA and the lipids making up the LNP with that of the HFA propellant along with the other excipients in the pMDI formulation would be of prime importance. One of the major formulation aspects that would need to be demonstrated in order for the suitability and feasibility of using the pMDI-HFA platform for the non-invasive targeted delivery of mRNA would be to demonstrate that the HFA propellant in the pMDI formulation does not significantly affect or alter the biological activity and structural integrity of the mRNA or any other nucleic acid dispersed or dissolved in it. This is because any biological or small molecule for it to be effective in a pMDI-HFA formulation needs to be first properly dispersed or dissolved in the HFA propellant in an optimally homogenous manner to result in reproducible dosing. The low dielectric constant of the HFA propellant combined with the high surface charge density of the dispersed biological molecule can cause agglomeration of dispersed particles in the HFA medium. Accordingly, the following sections of this review will discuss some of the previously published *In vitro* and *in vivo* studies involving the pMDI-HFA formulation of various biologic molecules like proteins, nucleic acids encapsulated or coated with surfactants, and phospholipids/LNPs, prior to describing their applicability to mRNA-1273 and BNT162b2.

Conti *et al*. [70] have shown *In vitro* that chitosan-coated plasmid DNA nanoparticles (CS-DNA NPs) when formulated as a suspension in a pMDI platform using HFA-227 as the propellant were able to transfect type II alveolar epithelial cell line A549 even after 6 weeks of storage in the propellant. Furthermore, the same study also reported that the CS-DNA NPs were also able to maintain their size, shape, and morphology even after 1 year of storage or incubation in the HFA227 propellant (at 298 Kelvin) when visualized using scanning electron microscopy (SEM). This implied that the DNA particles were able to withstand the high-pressure environment inside the pMDI canister. The study by Conti *et al*. [70] also reported high fine particle fractions of 57–65% and mass median aerodynamic diameters (MMAD) of about 2 microns indicative of the efficiency of the aerosolization process and its capability to deliver optimal DNA payload to the lungs. The authors had used pressure proof glass vials crimped with a 63-μl metering valve (EPDM, Spraymiser, 3 M Inc.) which are standard metering valves used in pMDI formulations.

Bains *et al*. [71] have reported similar *In vitro* results for the maintenance of structural and functional integrity of surfactant (lecithin)–coated plasmid DNA (synthesized by freeze drying) when the pDNA was formulated as a suspension in HFA-134a propellant using ethanol (8% v/v)

as a co-solvent. The authors have reported the successful transfection of A549 cells by the pDNA aerosolized from the prepared pMDI-HFA-based formulation with the aid of cationic lipids pre-dissolved in the cell culture media. The same authors also showed that the greatest number of DNA particles was found on stages four (cut-off diameter 2.1 microns) and five (cut-off diameter 1.1 microns) of the 8-stage Andersen Cascade Impactor (ACI) following aerosolization testing at a flow rate of 28.3 L per minute (l/min) indicating that most of the aerosolized DNA particles from the pMDI were in the respirable range. It is also to be noted that the device used by Bains *et al*. [71] consisted of an aluminum canister internally coated with fluorinated ethylene propylene and crimped with a Spraymiser™ 50-µl metering valve (3 M Drug Delivery Systems, Loughborough, UK). Thus, the choice of the metering valve with specialized internal coatings might also be an important formulation-controlled device parameter to be taken into account. In the study by Bains *et al*. [71], the importance of using the appropriate amount of surfactant to coat the DNA particles is also emphasized as they have shown that excessive surfactant concentration might result in lower DNA transfection efficiency as a result of the anionic surfactants like lecithin affecting the lipid-DNA complexation. Lipids are typically complexed with DNA particle for efficient uptake of DNA by the host cells. Furthermore, the surfactant concentration should also be kept within range so as to prevent DNA particle agglomeration and enable easy dispersion of agglomerated particle with the application of simple shaking force by hand. The use of ethanol as a co-solvent to enable the dissolution of the lecithin surfactant in the HFA-134a propellant is also well illustrated in this study by Bains *et al*. [71]. Although flocculation was observed by both authors Conti *et al*. [71] and Bains *et al*. [71], these floccules were reported to be easily re-dispersed upon minimal shaking of the formulation resulting in homogeneous dispersion of the DNA particles.

Ying Li *et al*. [72] have reported the successful formulation and delivery of bovine serum albumin (BSA) as a model protein using the pMDI-HFA134a platform [72]. The authors used prepared particles of BSA co-spray dried with sodium carboxy-methyl cellulose (Na-CMC) which were formulated as suspensions in HFA-134a without any other excipient-like ethanol or surfactant. In this study, Na-CMC was mainly used as a viscosity modifying agent for providing a solution suitable for spray drying and also to protect the BSA from thermal degradation during spray drying. When tested immediately after formulation on the twin-stage impinger, the Na-CMC free formulation was reported to show a FPF of 46% compared to a significantly higher FPF of 53% for the 80% w/w Na-CMC formulation. After storage for 5 months at room temperature, the Na-CMC free formulation showed a FPF of 35% compared to 48% for the

80% Na-CMC w/w formulation, thus suggesting superior FPF sustainability for the Na-CMC containing formulation as a function of storage time. Thus, irrespective of the presence or absence of Na-CMC, significant levels of FPF were obtained that were indicative of the possibility of optimal delivery of proteins to the lungs possible even without Na-CMC, although the addition of Na-CMC was shown to potentially result in improved and sustained FPF even after storage. Furthermore, the settling velocity, sedimentation, and re-suspension ability of the protein particles were shown to improve as a function of Na-CMC concentration in the formulation. Thus, the study by Ying Li *et al*. [72] demonstrated the feasibility for formulating proteins in HFA-134a propellant without any excipient and still obtain significant FPF using a BK357 30-µl metering valve. However, the study did not report any protein functional assays as direct evidences to support the maintenance of 3-dimensional integrity of the proteins (and thus their functional activity) post spray drying and post exposure to HFA-134a.

Quinn *et al*. [73] have reported that the Fourier transform (FT)-Raman spectrum of the protein lysozyme when suspended in HFA propellants (134a and 227) did not differ significantly in signal intensity and peak positions from that of the native protein molecule both in the solid and aqueous phase, in the primary regions of the FT-Raman spectrum. The results from Quinn *et al*. indicated that both the HFA propellants did not significantly alter the 3-dimensional structural integrity of the proteins suspended in them in the peptide backbone region, disulfide backbone, and C–C bonds. Oliver *et al*. [74] have also reported that the biological activity of DNAse I was unaffected by the HFA propellant even after storage for 6 months at −18°C, as shown by the maintenance of 3-dimensional structure of the protein in HFA medium. Similar results have also been reported by Brown *et al*. [75] wherein they have demonstrated the 6-month stability of the enzyme adenosine deaminase in a HFA propellant suspension when stored at 4°C/50% relative humidity and at 25°C/60% relative humidity.

Brown *et al*. [75] have reported the successful formulation of surfactant-coated bovine gamma globulin protein in other non-aqueous propellants like dimethylether and have reported optimum protein to surfactant molar ratios to be 1:1000 to 1:2000 suitable for obtaining optimal lung deliverable dose and product stability. Surfactant quantities lower than tested in the study were shown to cause protein agglomeration and poor aerosolization efficiency while very high surfactant concentrations resulted in dissolution of proteins in the propellant. In the same study, the authors have reported that the use of glass beads for agitation of pMDI formulation contents and inclusion of ethanol (2–4% w/w) resulted in increased respirable doses of the protein by virtue of improving reverse micelles formation. The protein is postulated to be encapsulated within the surfactant micelles.

The authors also showed that reducing the protein concentration from 2 to 0.2 mg/ml more than doubled the respirable fraction of proteins. However, the safety profiles of dimethylether compared to other FDA-approved HFA propellants are important points to take into consideration.

Williams III *et al*. [76] have also reported the development of a surfactant-coated bovine serum albumin (BSA) suspension formulation in HFA-134a using ethanol as a co-solvent. The BSA-surfactant complex was lyophilized to remove the water and the resulting solid powder was suspended in HFA-134a. The pMDI-HFA134a formulations were manufactured by the pressure filling method using silicone-lined glass aerosol vials (SGD Pharma, Paris, France) fitted with 150-μl metering valves (DF10 RC150, Valois of America, Inc., Greenwich, CT). Ethanol was used as a co-solvent to increase the solubility of the tested surfactants (Brij 97, Brij 98, Aerosol-OT (AOT), Tween 80) in HFA134a which is important to obtain a homogeneous distribution of surfactants in the propellant—a pre-requisite for obtaining sufficient dispersion of suspended proteins in the HFA134a. The authors have reported respirable BSA fractions in the range of 46–50% for Brij and Tween surfactants containing formulations at a surfactant/BSA ratio of 500:1 and ethanol concentration of about 7.89% (w/w). The study reported that lower levels of ethanol (1.58% w/w) were not sufficient to properly disperse the solid complex whereas higher levels of ethanol (11% w/w) were shown to result in reduced respirable or fine particle fraction due to increase in vapor pressure of the pMDI-HFA-ethanol blend. The use of ionic surfactant AOT was shown to result in lower fine particle or respirable fraction owing to the charge state of the surfactant in the propellant and also the DSC results showed that the ionic surfactant AOT decreased the stability of the protein whereas the inclusion of non-ionic surfactants like Brij and Tween increased the protein stability. Such surfactant-mediated protein deformation could be attributed to the charges of the proteins experiencing unbalanced electrostatic attractive and/or repulsive forces due to the surfactant charge. Therefore, the study by Williams III *et al*. [76] demonstrated that through the proper choice of surfactant, ethanol, and protein concentration combined with lyophilization prior to formulation of protein into HFA propellant, a stable pMDI-HFA-protein formulation can be obtained that can result in sufficient protein dosing to the lung.

Nakate *et al*. [77] have reported the formulation of a cyclopeptide drug FK224 complexed with beta-cyclodextrin as the solubility enhancer in chlorofluorocarbon (CFC) propellant blend (CFC11/CFC12/CFC114 in the ratio 17.5:65:17.5) containing 1% (w/v) lecithin as the dispersing agent. The authors have reported about 50% of respirable dose of FK224 achievable *In vitro* using such a pMDI-CFC model which was also shown to result in about 67% of the total inhaled dose reaching the lungs of healthy volunteers.

The authors had used a 20-ml aluminum canister (Bespak, UK) fitted with a 100-μl metering valve (Valois, France) and a type NK1 (Valois) mouth-piece for aerosolization which enabled emitted and respirable doses of the protein high enough to result in detectable protein levels in the plasma. Although Nakate *et al*. [77] have used CFC as the propellant which is not in use as per Montreal Protocol, the overall idea of delivering proteins using a pMDI format can be inferred from this study. The slightly lower vapor pressures of HFAs compared to CFCs combined with slower spray velocities possible for HFAs could possibly offset any hypothetical *In vitro* and/or *in vivo* differences in product performance.

Nyambura *et al*. [78] have formulated lysozyme protein nanoparticles complexed with lactose and coated with various surfactants like lecithin in HFA-134a and have shown that the biological activity of lysozyme was unaffected by the HFA134a as indicated by the unaltered UV absorbance of the protein at 450 nm, with 98% retention of biological activity. The authors also showed that the use of surfactants like oleic acid and span 85 (2% w/v) resulted in stable protein suspensions in HFA-134a that could be re-dispersed easily with gentle shaking. The very popular and also highly unstable human insulin has also been formulated as a suspension in HFA-134a propellant by Kos Pharmaceuticals (Weston, FL, USA) which has also been tested in type II diabetics to result in a rapid onset of action and comparable blood glucose levels to insulin glargine at base line and after 28 days when administered pre-prandially. This clearly showed that the structural and functional integrity of insulin was not compromised while remaining suspended in HFA134a [79].

Adjei *et al*. [80] have reported that the pMDI delivery of leuprolide acetate, a luteinizing hormone-releasing hormone (LHRH) agonist which is a small peptide only nine amino acids long, resulted in more than 50% of the inhaled dose getting deposited in the lung yielding a bioavailable dose that was greater than that achievable from nasal administration. Their study in healthy human males showed that the pMDI aerosol administration of leuprolide acetate using CFC11, CFC12, and span 85 resulted in significantly greater plasma AUC levels of the peptide compared to its nasal route of administration at all tested dose levels.

Therefore, there are multiple studies that have demonstrated the successful formulation of highly unstable biotherapeutics like nucleic acids, proteins, and peptides in HFA propellants showing the maintenance of the structural and functional integrity of the bio-therapeutics while formulated in various propellants. The stability of the biotherapeutics formulation in HFAs has also been shown to be sustained both with and without the aid of excipients like surfactants and co-solvents, depending on the nature of the physico-chemical interactions involved at the molecular level between the suspended biomolecules and the

HFA propellant molecules. In that perspective of the bio-therapeutic stability achievable with the HFA propellant as the dispersion medium or solvent, it should be plausible to expect similar or comparable maintenance of the stability of COVID-19 vaccines (mainly the SARS-CoV-2 viral spike protein mRNA) when formulated in HFA propellants. However, both the mRNA-1273 and BNT162b2 contain the mRNA encapsulated within LNPs and therefore, it would be of importance to consider the formulation of LNPs and other lipid-based vectors in HFA propellants.

Vyas *et al*. [81] have studied the *in situ* formation of liposomes encapsulating isoprenaline from the aerosolization of CFC propellant containing multiple dissolved lipid entities that commonly constitute the structure of LNPs. The authors have reported that the lipids and isoprenaline solubilized in CFC propellant formed liposome encapsulating isoprenaline *in situ* during and post aerosolization that were in the size range of 2.15–2.62 microns comparable to the size of liposomes prepared using injection methods. The authors have also demonstrated increased entrapment efficiency of isoprenaline as a result of including charged lipids that can bond with oppositely charged hydroxyl or amino groups of isoprenaline; such charge-based interactions have also been seen in the negatively charged mRNA bonding with the positively charged lipids making up the LNPs of the COVID-19 vaccines. The pMDI-CFC delivered liposomes were shown to sustain isoprenaline levels in lungs at more than 60% for 5 h post aerosolization indicating the potential for sustained delivery of bio-therapeutics using the tested pMDI dosage form. The liposomal-isoprenaline aerosol formulation was also shown to increase the half-life of isoprenaline in the isolated perfused rat lungs from 1 min (for the unencapsulated isoprenaline solution) to 5 h for the liposomal-isoprenaline formulation. Such sustained levels of bio-therapeutics in the lungs made possible using pMDIs could be beneficial from a clinical perspective to better treat COVID-19 by preventing further progression of the disease state to more serious conditions via targeted sustained pMDI-based vaccine delivery to the lungs.

Farr *et al*. [82] have investigated the *in situ* formation of liposomes following aerosolization of a pressurized formulation of phospholipids and concluded that the respirable fraction of liposomes is a function of lipid concentration, actuator orifice diameter, and vapor pressure of formulation. They have also demonstrated a formulation capable of delivering respirable fractions in the range of 20–45% without the use of any excipients like ethanol or surfactants that can add to improved stability and safety. The authors also confirmed the *in situ* formation of liposomes post aerosolization and deposition via scanning electron micrographs. Thus, this study is another example of how mRNA-LNP formulations can be initially dissolved in HFA propellants with or without any excipients, following which *in situ* formation of LNPs

(encapsulating mRNA) post propellant evaporation can be achieved to obtain respirable mRNA doses during actuation of the pMDI.

Apart from LNPs and other lipid-based vectors, the polymeric nanoparticles (PNPs) have also been widely studied for their capabilities to deliver bio-therapeutics. Therefore, it might also be possible to encapsulate the viral spike protein mRNA within PNPs which can then be formulated within a pMDI-propellant format for their targeted delivery to the lungs. Bharatwaj *et al*. [83] have shown that the dispersion of chitosan-poly(lactic acid) (PLA) nanoparticles with a core of pol(D,L-lactide-co-glycolide) (PLGA) in HFA227 resulted in maintenance of the structural and functional integrity of the PNPs as evidenced in their uptake by Calu-3 cells *In vitro*. Furthermore, the PNPs loaded with coumarin resulted in a FPF of 55% and MMAD of 2.1 micron (GSD 3.0) when formulated in HFA227 propellant and aerosolized using a 50-µl metering valve (EPDM spraymiser from 3 M) fitted with Ventolin HFA® actuators (GSK).

Thus, the formulation of lipid-based carriers and nanoparticles capable of encapsulating mRNA or any other biological entities in high vapor pressure propellants like HFAs has been demonstrated to be feasible in not only delivering reasonable respirable fractions of the encapsulated therapeutics but the stability of the lipid-HFA formulation has also been shown to be feasible. Therefore, the formulation of the LNP-based Moderna's mRNA-1273 and Pfizer BioNTech's COMIRNATY COVID-19 vaccines should technically be possible using the pMDI-HFA format. In that regard, a few of the formulation strategies applicable to the formulation of the currently FDA-authorized COVID-19 mRNA-LNP vaccines will be discussed [84].

The mRNA-LNP particles of the COVID-19 vaccines can be directly added into pMDI canisters of specific volume following which crimping of the pMDI canister using 50–100 µl metering valves can be done. The HFA propellant or their blends can then be pressure filled into the pMDI canister containing the mRNA-LNPs which can be expected to cause the dissolution of the lipids of the LNP causing the mRNA to be suspended in the matrix of HFA-lipid solution. The solubility of each of the lipids in the HFA propellant blends that form the LNP structure would have to be taken into account and depending on that either the addition of surfactants or ethanol as co-solvent will have to be investigated. Simultaneously, the stability of the mRNA in the chosen amounts of propellant and excipients would also need to be considered. Such a pMDI-HFA-lipid formulation containing the SARS-CoV-2 viral spike protein mRNA when actuated can be expected to form LNPs *in situ* that can assemble into solid lipid structures encapsulating the mRNA as observed in the study by Vyas *et al*. [81] and Farr *et al*. [82]. Such *in situ* formulation of mRNA-LNPs during and after actuation of the pMDI can be expected to result in the mRNA-LNPs

getting deposited in the different regions of the lungs. The *in situ* formation of LNP particles encapsulating the mRNA can also be investigated using appropriate laser diffraction studies or imaging studies to confirm the presence of LNPs post actuation of the pMDI formulation.

The direct addition of small volume (less than 1 ml) of an aqueous solution of the viral spike protein mRNA (without any lipids for encapsulation) into the pMDI canister followed by pressure filling the required quantity of HFA propellant is also a possible formulation route that can be inspired by the findings of the earlier mentioned studies. It can be expected that the actuation of such a pMDI-HFA-mRNA formulation although could deliver mRNA doses to the lungs, the free mRNA might not be taken up by the pulmonary cells for intracellular protein expression (due to their overall negative charge) and also might degrade in the lung biological milieu. The exposure of the free mRNA to the low dielectric constant HFA medium and the aerodynamic shear forces generated during aerosolization are important formulation and device-related factors that would need to be investigated in order to ensure that they do not have a significant impact on the structural and functional integrity of the formulated mRNA or any other biomolecule for that matter. Thus, the inclusion of lipids in the pMDI formulation might be an important excipient modification to not only maintain the structural and functional integrity of the suspended mRNA in the HFA pool, but also act as the vector for the successful internalization of the inhaled mRNA by the target pulmonary cells in the respiratory tract.

The challenges of forming a stable dispersion of mRNA-LNP particles in the HFA medium can be solved by various formulation approaches like coating the mRNA-LNP particles with surfactants like lecithin, Na-CMC, and cyclodextrins prior to their exposure to the HFA propellant. The addition of the aforementioned excipients can prevent the agglomeration of the mRNA-LNP particles in the HFA medium and improve the overall stability of the formulation as seen in the studies by Nyambura *et al*. [78], Nakate *et al*. [77], Brown *et al*. [75], and Williams III *et al*. [76]. The use of ethanol in the formulation to aid in the solubility of the surfactants in HFA could also be an important formulation strategy to consider in forming a stable formulation.

Following the formulation of the mRNA-LNPs in the HFA propellant medium, the major performance-related parameter that could be investigated is the respirable fraction and dose of the viral spike protein mRNA derivable from the chosen formulation strategy. The dose of mRNA or any other biomolecule in the respirable fraction delivered to the lungs should technically be high enough to trigger the mucosal immunity that can lead to secretion of IgA at levels higher than their IC50 against SARS-CoV-2 and its variants. A valuable *In vitro* study that could be conducted to confirm the successful performance of pMDI-HFA delivered mRNA-LNP would be to subject the cascade impaction-derived respirable fraction of the mRNA to internalization assays using specialized pulmonary cells (pre-infected and/or challenged later by viral loads) that are typically involved in the pathogenesis of COVID-19. The resulting secretion levels of antibodies by the cells and reduction in viral titers could be used as the metrics to assess the maintenance of the mRNA structural and functional integrity. However, the reports from experts in the inhalation product development field like Byron (1990) citing that the hydrophobic environments encountered in pMDI propellants do not necessarily denature the proteins and peptides is an encouraging observation in support of exploring the formulation of mRNA-LNP COVID-19 vaccines in a pMDI-HFA format [85]. A major reason that might be preventing the low dielectric HFA medium from significantly degrading the structural and functional integrity of biomolecules could be due to the inability of the solvent molecules to access the surface area of the biomolecules [86] which could again be specific to the biomolecule of interest.

For the device choice, the commonly used metering valves and mouth-piece actuators employed in the currently marketed pMDIs could be investigated for aerosol delivery of the HFA-mRNA-LNP. Metering valves with metering chamber volumes of 25–100 µl and made of various polymeric materials or stainless steel in combination with mouth-piece actuators with spray orifice diameters in the range of 200–300 microns and land length of 500–700 microns could be potential starting point devices to test in the formulation stage. The material of construction of the internal parts (like the spring and gasket) of the pMDI canister, metering valve, and mouth-piece actuator could also be an important parameter to investigate especially from the perspective of triboelectrification that can cause the materials and formulation to acquire electrostatic charges. Such electrostatic charge build up within pMDIs is a commonly encountered phenomenon that has been shown to be a function of material of construction of the device, moisture content, and also temperature (to name a few). Thus, the mRNA being a negatively charged species by nature could potentially adhere to internal cavities of the device that it could come in contact with during aerosolization as a result of electrostatic charge build up. Such mRNA-electrostatic charge interaction could lead to not only irreproducible dosing but also to possible interaction of device-generated electrostatic charges with the Van-der Waals and hydrogen bonding forces making up the structural and functional integrity of the mRNA's amino acid sequence.

The material of construction of the device should also be investigated for its potential to leach organic and inorganic substances into the pMDI's bulk mRNA-HFA formulation that can affect the formulation stability and performance. As an example, the leaching of silicone oil typically used as a

lubricant in the pMDI metering valve into the HFA formulation has been shown to cause drug particle agglomeration leading to the reduction of fine particle fraction. The choice of the device could also be based on the magnitude of the aerodynamic shear forces that can be generated during the aerosolization process that can disrupt the LNP structure exposing the mRNA prior to its deposition in the lungs.

From the clinical perspective, the success of pMDIs to target the COVID-19 mRNA vaccines to the intended regions within the lungs would be highly dependent on the breathing pattern of the COVID-19 patients which in itself could vary as a function of the disease severity. This is because of the synchronization of breathing and actuation of the pMDI required for optimal lung deposition of the inhaled dose with minimal possible oro-pharyngeal deposition. Furthermore, the pMDIs generally require the patient to inhale slowly and deeply with an additional 10 s of breathe hold for sufficient deposition of the inhaled dose in the lungs. Such a breathing pattern requirement however may not be possible for all the COVID-19 patient population mainly owing to the ARDS-triggered rapid and shallow breathing patterns typically seen in COVID-19 patients. Another important aspect is how well the time of injection of the bulk aerosol dose from the pMDI matches with the time of initiation of the patient's inspiratory cycle so that the majority of the emitted aerosol dose is injected just at the beginning of the inspiratory cycle which is an important condition to be satisfied for optimal lung deposition from pMDIs. Therefore, from a clinical perspective, it is important to ensure that the synchronization of the breathing initiation and aerosol bolus injection is possible or not for the COVID-19 patients before choosing the pMDI-HFA as the dosage form for the targeted delivery of COVID-19 vaccines.

Such rapid and shallow breathing seen in COVID-19 patients could further lead to increased respiratory rates (greater than 20 breathes per minute compared to 12–14 for normal adult) and altered tidal volume (about 400–500 ml compared to normal values of 1000 ml) [87, 88]. The increased respiratory rates can therefore cause higher air flow rates at the entrance of the oral cavity that could further reduce lung exposure of mRNA-LNPs inhaled from the pMDI due to increased turbulence being experienced by the aerosolized mRNA dose leading to increased oro-pharyngeal deposition. Furthermore, the increased airway resistance seen in COVID-19 patients presenting ARDS can also lead to increased turbulent flow and reduced laminar flow in the upper airways that could further lead to decreased peripheral deposition of inhaled drug from the pMDI [89, 90]. The rapid and shallow breathing that may not enable the patients to hold their breath for 10 s post inhalation might also result in decreased deposition of aerosols via sedimentation in peripheral lung regions [90, 91]. Therefore, the likelihood of COVID-19 patients with ARDS to inhale correctly from

a pMDI could be a challenging aspect from a clinical perspective which could lead to irregular dosing, in-adequate lung deposition, and ultimately treatment failure. Thus, even though the pMDIs can enable a feasible formulation of mRNA-LNPs in HFA propellants with or without any excipients, and even though a favorable MMAD and FPF for mRNA (encapsulated within LNPs) are possible from a pMDI-HFA format (as demonstrated in earlier described studies), the rapid and shallow breathing pattern combined with increased airway resistance and turbulence presented by the COVID-19 patients with ARDS might be the major hurdle in using the pMDI-HFA platform for the successful targeted delivery of COVID-19 vaccines to intended lung regions. The various studies discussing formulation of bio-molecules in a pMDI platform are summarized in Table I.

Formulation, Device, and Patient-Related Clinical Factors to Consider in the Development of DPI Versions of the COVID-19 mRNA Vaccines

Dry powder inhalers (DPIs) are the second most popular pharmaceutical inhalers with approximately 25% market share in the inhaler market [64]. Briefly, the principle of targeted delivery using a DPI is based on the exposure of a blend of active therapeutics with a carrier or excipient to the patient's inspired air leading to the de-aggregation of micronized active therapeutics from the carrier particles. The dispersed active therapeutic is expected to get deposited (governed by the drug particles' aerodynamic diameter) in the lungs and oro-pharyngeal cavities initiating the therapeutic action following the dissolution of the deposited therapeutics in the lung lining fluid. The different type of DPI formulations could be an adhesive mixture, nucleus agglomerate, or a spherical pellet depending on whether the carrier particles are micronized to the same extent as the active therapeutics and based on the uniformity of distribution of the active therapeutic moiety over the surface of the carrier particles [94]. However the most commonly encountered DPI formulation type in the commercially marketed inhalers is of the adhesive mixture type wherein the micronized APIs are attached primarily via Van der Waals forces to the surface of larger size lactose carrier particles. Based on the dependence of the DPI device on patient's inspiratory flow rate for sufficient de-aggregation of the API from the carrier excipient, the DPI devices can be broadly classified into active and passive devices. The active devices typically employ compressed air as an additional source of aerodynamic shear forces on top of the patient inhalation flow rate for the de-aggregation of the micronized API from carrier particles.

Although the DPI system as a whole is not completely immune to moisture ingress, DPIs are widely regarded to be better suited for delivery of highly unstable bio-therapeutics like proteins and peptides mainly owing to their inherent dry

Table 1 Summary of Studies that Have Investigated the Use of Various Pressurized Metered Dose Inhaler (pMDI) Formulations for the Targeted Delivery of Biomolecules

Name of study	Type of inhaler/device used	Formulation discussed	In vitro parameters and/or in vivo efficacy reported
Conti et al. (2021) [70]	Pressure proof glass vials crimped with a 63-µl metering valve (EPDM, Spraymiser, 3 M Inc.)	Chitosan-coated plasmid DNA particles suspended in HFA-227	MMAD of 2 microns, FPF 57–65% Successful transfection of A549 cells by the aerosolized DNA particles delivered from the pMDI formulation
Bains et al. (2010) [71]	pMDI consisting of aluminum canister internally coated with fluorinated ethylene propylene and crimped with a Spraymiser™ 50-µl metering valve (3 M Drug Delivery Systems, Loughborough, UK)	Surfactant (lecithin)–coated plasmid DNA suspended in HFA-134a-ethanol blend	8-stage ACI study at 28.3 l/min showed high dose of DNA deposited on stages 4 and 5 of the impactor Successful transfection of A549 cells by the aerosolized DNA particles delivered from the pMDI formulation
Ying Li et al. (2010) [72]	BK357 30-µl metering valve	Bovine serum albumin (BSA) co-spray dried with sodium carboxy-methyl cellulose (Na-CMC) suspended in HFA-134a	The inclusion of Na-CMC in the formulation was shown to result in improved FPF (53%) and stability for the BSA based on twin-stage impinger studies
Quinn et al. (1999) [73]	Not mentioned in the study	Lysozyme suspended in HFA 134a and 227	FTIR spectroscopy of the formulated lysozyme revealed that the peak positions and signal intensity were comparable to that of the native protein molecule
Oliver et al. (2000) [74]	Not mentioned in the study	DNAse formulated in HFA propellant	Biological activity of DNAse I was unaffected following storage in HFA propellant for 6 months at -18^{0}C
Brown et al. (2002) [92]	Not mentioned in the study	Adenosine deaminase enzyme in HFA propellant	The suspended enzyme was shown to be stable in the HFA propellant for 6 months when stored at different temperature and humidity conditions
Brown et al. (1996) [75]	MP-20CP metered-dose aerosol valves, Emson Research, Bridgeport, CT	Bovine gamma globulin in dimethyl ether using surfactants and ethanol as excipients	The effect of different excipients like surfactants and ethanol on the fine particle fraction has been reported
Williams III et al. (1998) [76]	Silicone-lined glass aerosol vials (SGD Pharma, Paris, France) fitted with 150-µl metering valves (DF10 RC150, Valois of America, Inc., Greenwich, CT)	Surfactant-coated bovine serum albumin suspended in HFA-134a using ethanol as a co-solvent	Fine particle fraction and protein stability was shown to be dependent on surfactant type, and concentration of surfactant and ethanol
Nakate et al. (2001) [77]	20-ml aluminum canister (Bespak, UK) fitted with a 100-µl metering valve (Valois, France) and a type NK1 (Valois) mouth-piece	Cyclopeptide drug FK224 complexed with beta-cyclodextrin as the solubility enhancer in chlorofluorocarbon (CFC) propellant blend (CFC11/CFC12/CFC114 in the ratio 17.5:65:17.5) containing 1% (w/v) lecithin as the dispersing agent	About 50% respirable fraction In vitro and almost 70% of the total inhaled dose of FK224 was shown to deposit in the lungs of healthy volunteers
Nyambura et al. (2009) [78]	Not mentioned in the study	Lysozyme protein nanoparticles complexed with lactose and surfactants like lecithin suspended in HFA-134a	UV absorbance of the suspended protein was shown to be unaffected by HFA propellant. The use of surfactants was shown to improve the re-dispersion of suspended particles
Hausmann et al. (2006) [79, 93]	Not mentioned in the study	Regular recombinant human insulin formulated in HFA-134a	In human subjects, rapid onset of action and comparable blood glucose level to insulin gargine was reported for the inhaled pMDI version of human insulin

Table I (continued)

Name of study	Type of inhaler/device used	Formulation discussed	In vitro parameters and/or in vivo efficacy reported
Adjei et al. (1992) [80]	20-ml Cebal aluminum cans with DF10/50-µl valves (Valois, Inc., Greenwich, CT)	Leuprolide acetate suspended or dissolved in trichlorofluoromethane and dichlorodifluoromethane	pMDI aerosol administration resulted in higher Cmax and AUC levels compared to intra-nasal route
Vyas et al. (1994) [81]	Not mentioned in the study	Isoprenaline solubilized in CFC propellant in the presence of lipids	In situ formation of liposome encapsulated isoprenaline having the potential for prolonging the levels of isoprenaline in the lung was reported
Farr et al. (1985) [82]	50-µl metering valve from Spraymiser, Neotechnig Engineering., UK	Lipids solubilized in chlorofluorohydrocarbon propellants	In situ formation of liposomes upon aerosolization of the pMDI. Respirable fraction of aerosolized liposomes was shown to be dependent on lipid concentration in the formulation and device parameters
Bharatwaj et al. (2010) [83]	Pressure proof glass vials fitted with 50-µl metering valves (EPDM spraymiser from 3 M) fitted with Ventolin HFA® actuators (GSK)	Polymeric nanoparticles suspended in HFA227	The pMDI formulations were shown to produce FPF of 50% and aerosols with MMAD of 2.1 microns. The aerosolized nanoparticles were also shown to be internalized by Calu-3 cells

state that precludes the presence of moisture in the formulation [95]. Such a relatively stronger fortress to moisture ingress (compared to pMDIs and nebulizers) could be attributed to the robust nature of the container closure system that come in immediate contact with the powder blend and also the DPI device compartments that serve as a safe locker for the blended powders. However, the blending process or any other manufacturing process of producing the blended powders could be susceptible to moisture ingress at a rate higher than that seen for pMDI pressure filling techniques. One of the major challenges in developing a DPI formulation for targeted delivery of bio-therapeutics is the need to obtain sufficient quantities of the micronized powder form of the biomolecule in the right geometric size. Further formulation challenges to overcome are the blending process of combining the micronized biomolecule with a carrier particle in order to produce a final blend of active-carrier entity with optimal intra-and inter-particulate cohesive-adhesive force balance that can sufficiently be overcome by the inspiratory force the target patient population can generate. The following sections will review several reported studies that have investigated the formulation of biomolecules and vaccines as dry powders suitable for inhalation along with the degree of success achieved in treating the targeted diseases employing them. Based on these previously reported studies, the current review would aim to propose various formulation and device-related strategies that could be investigated for enabling the targeted delivery of the two currently FDA-approved Moderna's mRNA-1273 and Pfizer BioNTech's COMIRNATY vaccines as dry powders.

Qiu et al. [96] have reported the development of a dry powder form of firefly luciferase mRNA using PEGylated cationic peptide, KL4 (a pulmonary surfactant protein B mimic) as the delivery vector combined with mannitol as carrier/bulking agent using spray drying and spray freeze drying for producing the dry powders. The KL4 cationic peptide was therefore used with an aim to immediately dissolve upon deposition in the lung lining fluids enabling the uptake of mRNA by the pulmonary cells. The SEM images of the resulting spray-dried and spray freeze–dried mRNA powders containing 5 µg of the mRNA were shown to be of spherical size with geometric particle diameter of less than 5 microns suitable for inhalation. The authors have reported that the spray-dried mRNA-PEG-KL4 formulation resulted in a fine particle fraction of 36–41%, whereas the spray freeze–dried mRNA-PEG-KL4 formulation yielded a FPF of 62–68% and MMAD was less than 6 microns for both formulations in the NGI study. The authors had used a size 3 hydroxypropyl methylcellulose (HPMC) capsule (Capsugel, West Ryde, NSW, Australia) containing the dry powders of mRNA loaded into the Breezhaler (Novartis Pharmaceuticals., Hong Kong). The higher fine particle fraction for the spray freeze–dried mRNA formulation was attributed to the

higher porosity of the spray freeze–dried powders that could have imparted better aerodynamic properties to them making them more suitable for deeper lung penetration in spite of their larger geometric size compared to the spray-dried particles. The authors have also reported that the PEGylation of the mRNA-KL4 peptide complex resulted in better uptake and transfection efficiency (almost fivefold) in both an *In vitro* A549 cell culture model and an *in vivo* mouse model. The PEGylation of the KL4 peptide was reported to have the effect of overcoming the poor aqueous solubility of the leucine rich regions of the KL4 peptide. The successful and safe transfection efficiency *In vitro* and *in vivo* showed that both the spray drying and spray freeze drying processes did not alter the structural and functional integrity of the mRNA.

Emig *et al*. [97] have recently investigated the *In vitro* and *in vivo* pulmonary delivery of dry powder version of a human monoclonal antibody AUG-3387 derived from the convalescent plasma of recovered COVID-19 patients. The AUG-3387 dry powder was manufactured using the proprietary thin-film freeze (TFF) technology and was shown to exhibit a fine particle fraction of about 50% and MMAD of 3.74 microns. The authors also demonstrated that the intra-tracheal administration of the AUG-3387 dry powders (0.3 and 1 mg/kg) to Syrian Hamsters after 24, 48, and 72 h of intra-nasal SARS-CoV-2 inoculation resulted in a dose-dependent 2.5- to fivefold reduction of the 5th day viral titers despite the fact that the treatment was delayed until 24 h post inoculation. The very interesting finding by Emig *et al*. [97] that was in favor of the inhaled DPI version of the AUG-3387 over the intra-peritoneal (IP) route was that the IP route required 3-times higher dose compared to the inhaled route to result in similar viral load reduction. Such a viral load reduction capability of the inhaled dry powder of AUG-3387 also showed that the drying process did not alter the structural and functional integrity of the monoclonal antibodies. The authors also demonstrated the efficacy of AUG-3387 against several of the mutant version of SARS-CoV-2 emergent until the publication date of their study including the highly contagious delta variant, with an EC50 of less than 200 ng/ml.

The targeted delivery of dry powders of biomolecules for the respiratory immunization against the influenza virus is a widely investigated area that can be highly relevant to the development of DPI versions of a COVID-19 mRNA vaccine. Audouy *et al*. [98] have shown that the pulmonary delivery of spray freeze–dried particles (using inulin as a cryoprotectant) of whole inactivated (WIV) live influenza virus to mice enabled the mice to develop sufficient immunity against a lethal challenge of intra-nasally administered specific strain influenza virus particles. The dry powder vaccine administration enabled the mice to produce both systemic IgG (log 10 titer of about 3.5) and mucosal IgA antibodies, with the IgA antibodies readily detectable both

in lungs and nasal cavity that was also nominally higher than the IgG titers obtained after intramuscular vaccination. The serum IgGs were also detected in the lungs following pulmonary administration of the spray freeze–dried vaccine. Furthermore, the weight loss seen in the mice 72 h following lethal challenge with the influenza virus was most pronounced in the control group (about 5%) compared to only about 1–2% seen in mice receiving the dry powder vaccine. The levels of neutrophils and macrophages in the broncho-alveolar lavage (BAL) were also readily detectable following inhalation of the dry powder vaccines. Furthermore, the viral titer load was also significantly lowered in the mice that inhaled the dry powder vaccine (log 10 value of about 4) compared to the control groups (log 10 value of 5.2) and intramuscular vaccination group (log 10 value of 4.8).

Patil *et al*. [99] have also reported that the pulmonary administration of spray freeze–dried whole inactivated influenza virus vaccine (5 µg dose) supplemented with monophosphoryl lipid-A (MPLA) to mice resulted in higher induction of mucosal and systemic immunity compared to pulmonary administration of spray freeze–dried vaccine without MPLA. Furthermore, the study also showed that the inhaled dry powder form of the MPLA-influenza vaccine was able to result in better protection when the mice were exposed to influenza virus as indicated by the reduction of viral titers in the lungs and weight loss prevention. The spray freeze drying process employing inulin as a stabilizer was reported to result in dry powder vaccine particles with aerodynamic diameters in the 1–5 microns (D10 0.7 micron, D50 1.731 micron, D90 3.5 micron) range which the authors claim to be suitable for respiration although no cascade impaction results were reported. The spray freeze drying process was also shown not to affect the structural and functional integrity of the vaccine particles as evidenced by intact virus structures in the SEM images and comparable hemagglutination assays. The addition of MPLA was shown to increase the respirable fraction of the dry powder vaccine by means of increasing the porosity of the spray freeze–dried particles while simultaneously not increasing the geometric particle size. Notably, the IgA levels in the broncho-alveolar lavage fluid, lungs, and nasal cavity were significantly higher for mice that received the dry powder MPLA-based vaccine compared to the intramuscular route indicating the better potential for the inhaled route to trigger the mucosal immunity.

Similar results for the efficacy of inhaled dry powder influenza vaccines were also reported by Amorij *et al*. [100] who investigated the respiratory immunization potential for the pulmonary delivery of dry powder influenza vaccine produced by spray freeze drying using inulin as a stabilizer. The authors reported that the dry powder influenza vaccine produced without any muco-adhesive adjuvants when delivered via the inhalation route to mice successfully triggered

significantly higher levels (by several folds) of cell-mediated (IFN-gamma and IL-4 producing T-helper cells), mucosal, and systemic immunity compared to the intramuscular route at similar dosing levels of 5 μg. The IgG and IgA levels in the lungs and serum were both significantly higher (by almost 50-folds) for the inhaled dry powder vaccine group compared to intramuscular route. Furthermore, the serum IgG levels remained higher at days 0, 28, and 42 for the mice that inhaled dry powder vaccines compared to the mice receiving vaccine via intramuscular route. The spray freeze–dried vaccine powders were also shown to result in 40% fine particle fraction from *In vitro* cascade impaction studies indicating significant vaccine dose capable of depositing in the lungs.

Smith *et al*. [101] have demonstrated the effectiveness of spray-dried lipid microparticles containing whole inactivated influenza virus in triggering mucosal immunity of the respiratory tract when administered as a dry powder to the lungs of rats/mice using an insufflator. The authors had used lipids like dipalmitoyl phophotidylcholine (DPPC) and distearoyl phosphatidylcholine (DSPC) that are biocompatible lipids present in lung surfactant to form the lipid microparticles of typical size between 1 and 5 microns suitable for oral inhalation and respiratory deposition. The authors have reported significantly higher levels for IgG titers (even after 28 days of initial immunization), IL-2, and IFN-gamma in the rats that received the spray-dried vaccines via the inhalation route compared to the rats receiving subcutaneous vaccines. Thus, the study by Smith *et al*. [101] depicts a scenario of lipid vector encapsulation of inactivated virus subject to spray drying to produce particles suitable for inhalation which gives an analogous formulation strategy to produce spray-dried particles of mRNA-LNP COVID-19 vaccines.

Lin *et al*. [102] have reported the development of dry powder measles vaccines suitable for respiratory delivery using the carbon dioxide (CO_2)–assisted nebulization with a bubble-dryer (CAN-BD) technique. They found that the dry powder measles vaccines when administered via the pulmonary route to rhesus macaques using the PuffHaler- and BD Solovent–induced measles virus specific humoral and T-cell response in addition to providing complete protection against the virus up to 1 year. The CAN-BD process was reported to produce dry powder particles predominantly in the 1–5 micron range suitable for pulmonary delivery and also did not affect the viability and potency of the measles vaccine which remained stable for at least 2 years when stored at 2–8°C. The use of Puffhaler was shown to deposit the vaccine mostly in the upper airways whereas the Solovent deposited the vaccine mostly in the lower airways as shown by the relative levels of the viral RNA in the tonsil and broncho-alveolar lavage (BAL) swabs, thus indicating a device-dependent regional lung deposition profile

for the aerosolized dry powder vaccine. The macaques that received the measles dry powder vaccine via the pulmonary route also showed higher levels of serum and mucosal antibody levels compared to the subcutaneous route at same dose. Specifically, the inhalation route also resulted in rapid higher levels of IgG, IgA, and IgM (that peaked at 8, 2, and 4 weeks respectively post immunization) compared to the subcutaneous route. Furthermore, the inhaled dry powder measles vaccines resulted in higher levels of IFN-gamma and IL-4 producing T-cells indicating higher cellular level immune response to dry powder vaccine administration. The dry powder vaccine was also shown to protect the animals against a wild-type strain of the measles virus challenged intra-tracheally 16-month post dry powder aerosol immunization as indicated by the negative RT-PCR results performed on the cells from nasal swabs and the high levels of T-cell immune response.

The *In vitro* solid-state characterization and respirable fraction analysis for the CAN-BD processed dry powder measles virus vaccine employing myo-inositol as the stability adding sugar has been reported by Burger *et al*. [103]. The authors reported that using an Aerolizer (Schering, Kenilworth, NJ), the cascade impaction studies showed a fine particle fraction of 50% (less than 5.8 microns) for the dry powder vaccine. The powder vaccine was also shown to maintain its moisture content (a key factor in vaccine stability and potency) below 0.5% which enabled a shelf-life of 2 years when stored at 2–8°C with the viral potency maintained at 1000 $CCID_{50}$ even after 2 years.

The *In vitro* and *in vivo* (animal studies) results showing the positive results for the inhaled dry powder measles vaccine reported by Burger *et al*. [103] and Lin *et al*. [102] were also further reflected in the phase 1 study by Agarkhedkar *et al*. [104] who have reported the safety and efficacy of the inhaled dry power measles vaccines in humans. The authors showed that the subjects who received the dry powder vaccine via the Puffhaler and Solovent not only tolerated the vaccine dose with no adverse events (neither viral virulence nor neurological risk) but also developed significantly higher serum IgG levels compared to the subjects receiving subcutaneous vaccines as measured on days 7, 28, and 84 post vaccination. Thus, the dry powder version of measles vaccines developed using the CAN-BD technique was shown to be effective in producing thermodynamically stable vaccines with respirable fractions sufficient enough to elicit protection against the measles virus both in animals and humans.

Tran *et al*. [105] have investigated the *In vitro* properties of chitosan nanoparticles containing pDNA produced using the spray freeze drying technique and reported that the dry powders resulted in a sustained release profile of pDNA (about 60% cumulative pDNA release over 90 days) with fine particle fractions of 20% and MMAD of 1.7 micron suitable for lung deposition. Thus, the study by Tran *et al*. [105]

demonstrated the advantage of an inhaled dry powder containing biomolecule that can enable sustained release over a long period of time which would fit well into the vaccination protection expected from a COVID-19 mRNA vaccine expected to last for a long time. The pDNA nanoparticles were also reported to lose 10–20% of activity after storage at 4 and 25°C respectively for 3 months indicating that they can retain 90% of their activity for a reasonable period without requiring ultra-cold storage conditions as required for the currently approved mRNA-LNP-COVID-19 vaccines.

Cheow *et al*. [106] have reported the superiority of the spray freeze drying process over traditional spray drying to produce dry powders of thermally unstable poly(caprolactone) (PCL—melting point 62°C) nanoparticles encapsulating levofloxacin using polyvinyl alcohol (PVA) and mannitol as the excipients. This is because typically spray drying is done at inlet temperatures of around 100°C to achieve a faster convective drying rate needed to obtain low density powders with aerodynamic diameters suitable for lung deposition, whereas in spray freeze drying, the typical temperatures encountered by the actives are in the ultra-low range of − 196 to 0°C and conditions are maintained below the triple point of water (6 mbar, 0.01°C) for production of dry powders. Thus, spray freeze drying offers the possibility to manufacture dry powders of biomolecules without employing high temperatures, making it suitable for thermally unstable molecules. However, the use of excipients is important in SFD process as cryo-protectants, and to facilitate re-dispersion of spray freeze–dried nano-aggregates to primary nanoparticles upon coming in contact with the lung lining fluid. Although this study did not employ a biological entity as the active, the study demonstrated the superiority of spray freeze drying over spray drying for formulation of thermally sensitive therapeutics as dry powders for inhalation. The authors were able to produce particles with mean geometric diameters of 18 microns smaller than the secondary bronchial diameter with aerodynamic diameters in the range of 4–5 micron owing to the high porosity (result of using mannitol as the excipient) that made the particles suitable for lung deposition in spite of their large geometric size. The cascade impaction studies demonstrated 94% of loaded dose emitted but with a low fine particle fraction of 10–14% when tested using a standardized powder entrainment tube (SPET) at 85 l/min flow rate, which was attributed to agglomeration of nano-aggregates upon aerosolization.

Fiedler *et al*. [107] have shown that both spray drying (inlet 60°C and outlet 29°C) and spray freeze drying can be used to produce dry powder versions of three interleukin-8 based proteins (7–74 kDa) that were shown to be stable for up to 12 weeks of storage without the use any adjuvant or any specialized excipients. Although the authors have reported that there was no alteration in the structural and functional integrity of the spray-dried or spray freeze–dried interleukins, the geometric diameters of the particles were shown to be greater than the range suitable for inhalation primarily due to particle agglomeration. Furthermore, the study also did not involve any cascade impaction techniques to assess the respirable faction of the spray-dried and spray freeze–dried powders. The absence of excipients in the formulation and chosen spray atomization flow rate could have caused particle agglomeration leading to higher particle size distribution. Nevertheless, the study by Fiedler *et al*. [107] demonstrated that spray drying and spray freeze drying when conducted at optimal conditions can be used for production of stable dry powder versions of biomolecules.

Lo *et al*. [108] have reported that the spray freeze drying of bovine serum albumin (BSA) using hydroxypropyl-β-cyclodextrin (HPβCD) as the stabilizer resulted in spherical and porous particles and fine particle fractions in the range of 50–80% (using Breezhaler®) alongside MMADs in the range of 1–2.5 microns. The authors also concluded that among the process parameters investigated the atomization gas flow rate had the maximum effect on the aerosolization properties of the spray freeze–dried particles compared to solute and BSA concentration in the feed stock. The study reported that higher atomization flow rates resulting in reduced particle size and higher FPF. However, the high atomization flow rates were also associated with particle aggregation. The study also showed that the spray freeze–dried BSA was structurally stable as assessed by SDS-PAGE.

Liang *et al*. [109] have used spray freeze drying to produce dry powder DNA and siRNA particles using mannitol as the bulking agent that resulted in fine particle fractions of 15–30% (DNA) varying directly as a function of DNA feed concentration and about 18% FPF for the siRNA using a low resistance Breezhaler device. The spray freeze–dried particles had the geometric size in the median range of 8–12 μm which although relatively large compared to typical inhalable particles size range was balanced by the high porosity of the particles that enabled 15–30% FPF.

Otake *et al*. [110] have reported the use of spray freeze drying of an aqueous formulation containing multiple hydrophobic amino acids using sodium fluorescein as the model active. The dry particles obtained were shown to be spherical with high porosity and geometric mean diameters in the range of 5–10 microns. The study by Otaka *et al*. (2016) also showed that particles produced using glycine as the excipient had the highest geometric particle size and lowest fine particle fraction (20%), whereas the L-phenylalanine-based particles showed the lowest geometric size but highest fine particle fraction (80%). The other amino acid groups used in the formulation resulted in FPFs in the range of 40–75%. The study also demonstrated that the use of amino acids (especially iso-leucine, leucine, and L-phenylalanine) not

only improved fine particle fraction but also enabled anti-hygroscopic properties to the overall bulk of the dry powder by resisting moisture ingress for up to 4 weeks.

Milani *et al.* [111] have investigated the use of hydroxyl-propyl beta-cyclodextrin (HPβCD) as an adjuvant protein stabilizer for the spray freeze drying of IgG antibodies in the presence of sugars like trehalose for synergistic protection to the protein. The spray freeze–dried particles were shown to be spherical and porous with geometric particle size in the range of 6–12 microns. The authors showed that the aggregation rate and stability of the IgG dry powders were dependent on the amount of trehalose and (HPβCD) in the stock feed. The most stable IgG spray freeze–dried powders were shown to result in a fine particle fraction and emitted dose in the range of 48–57% and 90–93% respectively using the Cyclohaler®. Thus, irrespective of the relatively larger geometric size, the porosity of the spray freeze–dried particles enabled a sufficiently high fine particle fraction to the dry IgG powders. An earlier study from the same research group by Pouya *et al.* [4] showed that the incorporation of trehalose: HPβCD was shown to result in the best protection to spray freeze–dried IgG particles while at the same time sustaining the fine particle fraction at more than 50% using the Cyclohaler ®

Liang *et al.* [112] have reported the use of spray drying and spray freeze drying for the production of pH-sensitive peptides complexed with DNA (at 10:1 w/w ratio) using mannitol as the carrier. The authors reported about 83% DNA recovery for spray freeze drying and 76% for spray drying and although both drying process did not alter the structural integrity of the DNA as indicated by gel retardation assays, it was seen that both the drying process resulted in modification of the original super coiled structure of the DNA to the more relaxed form. The spray-dried particles were smaller with D50 around 1.8 microns but not porous and also morphologically different from that of the highly porous spray freeze–dried particles that were larger (D50 about 10 microns). The FPF for the spray freeze dried although nominally higher was found to be almost the same as compared to the spray-dried formulation at about 50%. The authors also reported that while both spray drying and spray freeze drying did not alter the structural and functional integrity of the DNA complexes, the spray-dried DNA complexes were shown to result in higher transfection efficiency (in A549 cell models) compared to both spray freeze–dried and freshly prepared DNA complex. The higher transfection efficiency for the spray-dried DNA powders was attributed to the possible difference in the cellular mechanism of uptake arising as a result of differences in particle morphologies. While the presence of mannitol in the formulation was shown to result in a dose-dependent increase in transfection efficiency owing to its ability to open up cellular channels for transport, both the drying process were prone to reduction in transfection efficiency when exposed to pulmonary surfactant containing broncho-alveolar lavage fluid.

The same group of authors (Liang *et al.* [113]) have also showed in another study the successful use of spray drying (inlet and outlet temperatures of 50°C and 34°C) for the production of dry powder siRNA electrostatically coupled to pH-sensitive peptides like histidine or 2,3-diaminopropionic acid and employing mannitol as a bulking agent. The authors used the low resistance Aerolizer® and showed that the *In vitro* fine particle fraction was 40% using the next-generation impactor operated at 100 l/min for 2.4 s. The particle size distribution for the siRNA dry powder complex as measured by laser diffraction was shown to be in the range of 1.4 to 1.8 microns (D50) with D90 in the range of 2.7 to 3.5 microns. The SEM images also showed spherical particles with wrinkled surface although no reports were made on their porosity. The spray-dried siRNA-peptide complexes were also shown *In vitro* to successfully transfect A549 and MDCK cells with both type of transfected cell lines showing significantly higher resistance to lethal influenza viral challenge as implicated by more than 10,000-fold reduction in viral titers in A549 cells compared to negative controls. The authors also reported that the transfection efficiency of the siRNA dry powders in the presence of broncho-alveolar lavage fluid (BALF) was dependent on the nature of peptide used for forming the complex with the siRNA. Such peptide-dependent siRNA transfection efficiency highlighted the function of the peptides to form stronger bonds with the siRNA necessary to overcome the degradative nature of the zwitterionic phospholipids present in BALF. Thus, the spray-dried process employed by Liang *et al.* was shown to be capable of producing stable siRNA dry powder suitable for inhalation from a low resistance inhaler, with the biomolecule's structural and functional integrity intact. The structural and functional integrity of the siRNA was also shown to be unaltered by the drying process as indicated by the transfection efficiency and reduced influenza viral titers 48 h post viral challenge.

Chow *et al.* [114] have reported the use of spray drying to make dry powders of siRNA and DNA using mannitol as bulking agent and stabilizer along with L-leucine as the dispersion enhancer. The inclusion of L-leucine was shown to reduce the geometric particle size along with MMAD and specifically the formulations containing L-leucine and mannitol in 1:1 ratio were reported to result in particle morphologies more suitable for inhalation compared to other groups. The geometric particle size (D50) of spray-dried DNA particles were shown to vary between 2.2 and 3 microns while that for spray-dried siRNA particles varied between 2 and 8 microns. The inclusion of L-leucine in 1:1 ratio was shown to result in higher FPF of about 45% for the siRNA and about 55–60% for the DNA formulations using the Breezhaler®. The gel retardation assay showed that the

siRNA integrity was unaltered by the spray drying process performed at inlet temperature of 80°C and outlet temperature of 49–52°C.

Okuda *et al.* [115] have reported the use of supercritical carbon dioxide–based antisolvent precipitation technique to prepare dry powder of firefly luciferase gene siRNA which was shown to be effective when administered to mice via the trachea. Although the supercritical fluid technique resulted in long needle shaped particles, the authors have reported that the manual grinding of the particles enabled them to be suitable for intra-tracheal administration by achieving a particle size of 10–20 microns. The supercritical fluid technique and the manual grinding were also shown to not alter the structural and functional integrity of the siRNA as evidenced by gel electrophoresis. The authors have also reported that the use of chitosan as an excipient retained the siRNA in the lungs and delayed its translocation to other organs in the mice indicative of chitosan's benefit in sustaining lung delivered doses of the siRNA. Furthermore, the authors have also reported that the molecular weight and degree of de-acetylation of chitosan to play an important role in the siRNA transfection efficiency. The intra-tracheally administered siRNA powders were also shown to inhibit the luminescence associated with the tumor growth induced by colon26/Luc cells in the lungs of the mice to a significantly higher degree compared to untreated mice, indicative of the successful transfection and gene silencing in lung epithelium. However, the study did not report any *In vitro* cascade impaction techniques in order to conclusively comment on the lung deposition efficiency, if the particles were to be inhaled from a DPI device.

Other studies employing supercritical carbon dioxide–based antisolvent precipitation techniques for the preparation of inhalable biomolecules have also been reported by Todo *et al.* [116] who have compared the supercritical fluid technique to spray drying for the preparation of inhalable insulin particles using mannitol as the carrier. The *In vitro* cascade impaction tests using the Jethaler (Hitachi Unisia Automotive., Ltd., Atsugi., Japan) showed that the use of the supercritical fluid–based technique resulted in MMAD in the range of 3.2 microns whereas the spray-dried powders showed a MMAD of about 4.7 microns. The authors have also reported that the supercritical fluid technique resulted in higher fine particle fraction (about 48%) compared to the spray-dried powders (about 30%) as seen in the *In vitro* cascade impaction tests. Furthermore, the SEM images were shown to depict a rectangular morphology for the insulin powders produced using the supercritical fluid technique compared to spherical particles obtained from spray drying which might have important implications to the inter-particulate packing when loaded into the capsule of the DPI device and also while

emptying from the DPI capsule upon inhalation. The study by Todo *et al.* [116] also reported that the insulin powders prepared by supercritical fluid technique were superior in their *in vivo* efficacy to reduce blood glucose level following intra-tracheal administration compared to the spray-dried counterpart. Thus, the supercritical fluid–based technique was shown not to alter the structural and functional integrity of the insulin even after the drying process.

Thus, there are multiple studies that have utilized different types of manufacturing techniques like spray drying, spray freeze drying, and supercritical fluid–based precipitation to produce dry powder version of biomolecules for their targeted delivery to the lungs. Such techniques are applicable to the production of dry powders of vaccines suitable for their targeted delivery to the lungs via the oral inhalation route. Therefore, the current FDA-approved COVID-19 vaccines can be reformulated as dry powder versions using the commonly used techniques like spray drying, spray freeze drying, and supercritical fluid technique describe in previously reported studies. In that perspective, the following sections of the review will summarize brief description of some of the formulation and device-related strategies applicable to the development of inhalable dry powder versions of the COVID-19 vaccines taking the currently FDA-approved COVID-19 vaccines as prototypes. The techniques described in the following sections are only hypothetical experimental designs pointing in the possible directions any targeted vaccine delivery program can take and subject the models to further *In vitro* and *in vivo* validation as applicable to various stages in vaccine development.

Taking the Moderna's mRNA1273 vaccine as the example, we see that the formulation is developed as an aqueous-based vaccine suspension suitable for intramuscular administration with water for injection (WFI) as the dispersion medium. The LNP-encapsulated mRNA is suspended in the pool of WFI along with other dissolved excipients of which sucrose constitutes to the maximum weight percentage. The main purpose of including sucrose is to act as a cryopreservant to protect the LNPs and the mRNA from the recommended extremely low storage conditions. However, such a cryoprotective effect may not be required for dry powder version of the mRNA vaccine since extreme low temperature storage conditions may not be required to maintain the stability of the dry powder dosage forms in general, although refrigerated conditions or $-20°C$ might be applicable. Therefore, for the production of dry powder version of Moderna's mRNA1273 COVID-19 vaccine, sucrose may not be required as an excipient but rather bulking agents like mannitol or lactose can be added to the aqueous phase to serve as major excipient carrier particles as is typical of dry powder formulations for inhalation. A similar approach

can also be taken for the Pfizer BioNTech's COMIRNATY vaccine since it also contains LNP-encapsulated mRNA in an aqueous suspension as the basic formulation design with high percentage of sucrose as a cryopreservant.

A typical starting point in the pre-formulation step (prior to drying process) would be similar to a thin-film evaporation process involving the preparation of aqueous solution of the mRNA along with bulking agents like mannitol or lactose to make up the aqueous phase. Separately, the lipids making up the LNPs could be dissolved in an organic solvent which can later be subjected to evaporation to result in a thin lipid film in the container. To this thin lipid film, the aqueous phase containing the mRNA and mannitol or lactose excipients can be added in a controlled manner to form the aqueous suspension of LNP-encapsulated mRNA vaccine. Thus, the same encapsulation process used in the production of LNP-loaded mRNA vaccine for intramuscular administration can be utilized but with lactose or mannitol replacing the sucrose. The LNP-mRNA suspension in the aqueous base containing the mannitol or lactose can then be used as the stock feed solution for spray drying, spray freeze drying, or supercritical fluid technique resulting in the production of dry powder particles containing the LNP-encapsulated mRNA vaccine embedded in a solid powder matrix of mannitol or lactose similar to the dry powders obtained in the earlier mentioned studies.

Inclusion of non-viral vectors like chitosan in the formulation can also be a possible formulation strategy to enhance the uptake of the mRNA vaccine and its stability as shown by Okamoto et al. [117]. This can be attributed to the enhanced cellular uptake processes like endocytosis that could specifically target chitosan-tagged molecules resulting in their higher uptake. However, the toxicity and comparative studies with a negative control in the absence of viral vectors can also be used to decide on the inclusion of non-viral vectors for enhanced uptake since the LNPs themselves might also be subject to endocytosis. The interaction between LNPs and chitosan or other non-viral vectors in the formulation might also have to be taken into account in deciding whether or not to include non-viral vectors in the light of common cellular uptake pathways possible for LNPs and non-viral vectors which could lead to competitive inhibition of uptake of both entities in a non-synergistic type of interaction between LNPs and non-viral vectors.

The aqueous formulation containing LNP-encapsulated mRNA along with other excipients like mannitol or lactose can directly be subject to the spray drying process with the inlet and outlet temperatures being optimized as per the thermal stability of the most heat-sensitive component keeping the melting points of the formulation constituents in mind. The drying process parameters that can be optimized for desirable results could be the concentration of mRNA, lipids, and other excipients like mannitol or lactose in the stock feed along with instrument parameters like inlet and outlet temperature, stock feed rate, and shape of nozzles. The drying process and instrument parameters can be optimized as per the solid-state properties of the dry powder mRNA-LNP particles and their corresponding melting and denaturing temperatures. The dry powder version of the mRNA-LNPs can be mainly characterized as per their aerodynamic performance using cascade impaction tests to see if sufficient quantity of the vaccine can be deposited in the lungs enabling required levels of different types of antibodies being secreted in the lungs.

As much as it is important to devise a robust and optimal formulation strategy for the production of a dry powder version of a COVID-19 vaccine, it is equally important to lay out the strategy for the optimal device selection based on clinically relevant parameters as applicable to the COVID-19 patients. This is because the success of any inhalation treatment using any pharmaceutical inhaler is well known to depend on the intricate relationship between the formulations, device, and patient-related clinical factors that are interdependent on each other. Therefore, the choice of the dry powder inhaler device to be used for inhalation would have to be considered based on the breathing patterns characteristics of COVID-19 patients which could again vary as a function of the intensity and stage of the infectious disease. A significant percentage of COVID-19 diagnosed patients have been reported to present with shortness of breath with dyspnea being a common symptom across disease severity groups especially in moderate and severe cases. The respiratory rates have been reported to be greater than 30 breathes per minute characterized by rapid and shallow breathing in both moderate and severe COVID-19 cases that can mainly be attributed to development of ARDS as the virus infiltrates into the alveolar space and also into the respiratory center of the brain [118].

Thus, the rapid and shallow breathing characterized by higher respiratory rates seen in COVID-19 patients compared to the normal breathing conditions is an important clinical factor to take into consideration in the choice of a suitable DPI device. As is already known, most of the DPIs have an intrinsic resistance that needs to be overcome by the patient's inspiratory force so that sufficient aerodynamic shear forces can be generated during inhalation that is needed for the optimal de-aggregation of the active moieties from the carrier particles for optimal lung deposition of inhaled therapeutics. Thus, the clinically relevant dyspnea type of breathing factor need to be combined with the DPI's intrinsic resistance value in the choice of a DPI for the targeted delivery of the COVID-19 dry powder vaccines to the lungs. This is because most of the current DPI devices have been widely used for the treatment of COPD and asthma-related lung disorders

whereas the use of DPI for targeting COVID-19 vaccines would require the revisiting of the current DPI designs or may be even devising newer DPI designs more suitable for the peak inspiratory flow rates and inspiratory volumes seen in COVID-19 patients [119, 120]. The ability of the COVID-19 patients to generate a rapid and deep breathing pattern as required by most of the current DPI designs in order to overcome the device resistance would thus plays an important role in the targeted delivery of dry powder COVID-19 vaccines to the lungs. The various studies discussing dry powder particles of biomolecules are summarized in Table II.

Conclusion

The major route of SARS-CoV-2 and its variants entry into the human body has been well established to be the nasal and oral inhalation route with the lungs being the primary organs of destruction and major sites of viral proliferation leading to cytokine storm–mediated ARDS. Furthermore, the very high expression of ACE2s on the mucosal surface of the human respiratory tract makes the respiratory tract more vulnerable to viral infiltration. Therefore, triggering the mucosal immunity via targeted delivery of COVID-19 vaccines to the lungs can be expected to result in better prophylactic protection from the virus and its variants compared to intramuscular administration of the vaccines. The targeted delivery of the current FDA-approved LNP-based mRNA vaccines from Moderna (mRNA1273) and Pfizer BioNTech (COMIRNATY) to the lungs via pMDI- and DPI-based formulations could be important feasible options to explore in order to combat emergent strains of the virus. As inferred from previously reported studies on inhalable biomolecules, the mRNA-LNP-based COVID-19 vaccines might have few physico-chemical properties suitable for their formulation in HFA propellant–based (pMDI) and as dry powder–based (DPI) dosage forms.

The inherent lipid soluble nature of the lipid constituents of the LNPs in mRNA1273 and COMIRNATY is an important physico-chemical property that can enable the formulation of the lipids as a solution in the HFA propellant along with the viral mRNA remaining suspended in the external pool of lipid-HFA. Previous studies investigating the pMDI-HFA-based formulations of biomolecules have reported the feasibility of formulating lipids as solutions in HFA along with the other hydrophilic biomolecules remaining suspended and well dispersed in the external HFA pool. Thus, the pMDI-HFA-based platforms might rely largely on the ability of the dosage form to result in the *in situ* particle formation of LNPs encapsulating the mRNA as a result of the flash evaporation of the propellant.

For the purpose of dry powder COVID-19 vaccine formulation, the fundamental aqueous nature of the LNP-mRNA suspension of the mRNA1273 and COMIRNATY vaccines can be considered a suitable physico-chemical property that can enable them to be used as feed stock (starting material) for various drying processes like spray drying, spray freeze drying, and supercritical fluid precipitation techniques without any major formulation modification. In anticipation of the dry powder versions of the vaccines not requiring ultra-low storage temperatures, the sucrose in the currently approved intramuscular formulation could be replaced with mannitol or lactose as bulking agents that can also serve as carrier particles for the dry powder version of the COVID-19 vaccine. Thus, there is reasonable evidence from previous studies investigating inhalable biomolecules and based on the major inherent physico-chemical properties of the current FDA-approved intramuscular COVID-19 vaccines to support the feasibility of developing pMDI and DPI versions of the vaccines. While it might also be feasible for the maintenance of structural and functional integrity of the mRNA when formulated as pMDI and DPI dosage forms, it might be important to investigate the maintenance of such structural and functional integrity of the mRNA vaccine for longer periods of time in order to allow sufficient time for shipment and storage of the vaccine.

The major clinical challenge in the development of pMDI and DPI formulations of the mRNA-1273 and COMIRNATY could be encountered in ensuring the suitability of the inhaler device for the COVID-19 patients characterized with ARDS-related rapid and shallow breathing. This is because most of the pMDIs are generally regarded to require a slow and deep breathing (with breathe hold) and the DPIs are known to require sufficient inspiratory force to overcome their inherent device resistance. The breathing difficulties and increased respiratory rates seen in COVID-19 patients might present a significant clinical challenge in the development of a suitable pMDI and DPI versions of the COVID-19 vaccines. In that perspective, the breath actuated devices might be better suited for the targeted delivery of COVID-19 vaccines to the lungs. Thus, overall while the formulation of mRNA-1273 and COMIRNATY as pMDI and DPI dosage forms might be feasible, the success of such an inhaled version of the COVID-19 vaccine might ultimately depend on the matching of the breathing pattern of COVID-19 patients with the inhaler's breathing requirements. The different formulation, device, and clinical strategies involved in the development of a nebulizer-based dosage form for the COVID-19 vaccines will be covered in part II of the review.

Table II Summary of Studies that Have Investigated the Use of Various Pressurized Metered Dose Inhaler (pMDI) Formulations for the Targeted Delivery of Biomolecules

Name and year of study	Type of inhaler/device used	Formulation discussed	In vitro parameters and/or in vivo efficacy reported
Qiu et al. (2019) [96]	Size 3 hydroxypropyl methylcellulose (HPMC) capsule (Capsugel, West Ryde, NSW, Australia) containing the dry powders of mRNA loaded into the Breezhaler (Novartis Pharmaceuticals, Hong Kong)	Firefly luciferase mRNA using PEGylated cationic peptide, KL4 (a pulmonary surfactant protein B mimic) as the delivery vector combined with mannitol	Fine particle fractions in the range of 40–68% were shown from NGI studies Successful transfection of A549 cell models and in mice, by the dry powder mRNA formulation was also reported
Emig et al. (2021) [97]	Size 3 hydroxypropyl 235 methylcellulose (HPMC) capsules (Vcaps® plus, Capsugel®, Lonza, Morristown, NJ, USA), loaded into a high-resistance Plastiape® RS00 inhaler (Plastiape S.p.A, Osnago, Italy)	Dry powder version of a human monoclonal antibody AUG-3387 derived from the convalescent plasma of recovered COVID-19 patients using mannitol/leucine or trehalose/leucine as carrier	Dry powders of the monoclonal antibody had MMAD 3.74 microns and FPF of 50% Intra-tracheal administration of monoclonal antibody dry powders was shown to result in a dose-dependent reduction of lung viral titers in Syrian Hamsters
Audouy et al. (2011) [98]	DP-4 M dry powder insufflator (Penn Century Inc., Philadelphia)	Spray freeze–dried whole inactivated live influenza virus using inulin as stabilizer	The vaccine powder when administered to mice via pulmonary route was shown to trigger both mucosal and systemic immunities sufficient for protection against lethal dose of influenza virus
Patil et al. (2013) [99]	Dry powder insufflator (Penn Century Inc.)	Spray freeze–dried whole inactivated influenza virus vaccine (5 µg dose) supplemented with monophosphoryl lipid-A (MPLA)	Dry powder vaccine particles were reported to have aerodynamic diameter in the 3 micron range (90 percentile) The inhaled vaccines were shown to result in high mucosal immunity in mice compared to other routes of administration
Amorij et al. (2007) [100]	DP-4 M dry powder insufflator (Penn Century Inc., Philadelphia)	Spray freeze–dried influenza virus using inulin as stabilizer	In vitro FPF of 40% from cascade impaction studies Inhaled vaccine triggered higher levels of mucosal and systemic antibodies compared to intramuscular route in mice
Smith et al. 2003 [101]	Dry powder insufflator (Penn Century Inc., Philadelphia)	Spray-dried lipid microparticles containing whole inactivated influenza virus particles	Inhaled vaccines were reported to result in higher IgG levels in rats compared to subcutaneous route
Lin et al. (2010) [102]	PuffHaler (Akitv-Dry and RPC Formatec) and BD Solovent (Beckton Dickinson BD Technologies) for animal studies	Dry powder measles vaccine prepared by carbon dioxide–assisted nebulization	Pulmonary route of vaccine administration to rhesus macques was shown to result in higher levels of both mucosal and serum antibodies compared to subcutaneous route at similar doses
Burger et al. (2008) [103]	Aerolizer (Schering, Kenilworth, NJ) for the In vitro cascade impaction study	Dry powder measles vaccine prepared by carbon dioxide–assisted nebulization	In vitro FPF of 50% based on cascade impaction studies was reported
Agarkhedkar et al. (2014) [104]	PuffHaler (Akitv-Dry and RPC Formatec) and BD Solovent (Beckton Dickinson BD Technologies)	Dry powder measles vaccine prepared by carbon dioxide–assisted nebulization	Inhalation of the dry powder measles vaccines by human subjects resulted in higher mucosal and serum immunity compared to subcutaneous injection
Tran et al. (2020) [105]	Standardized powder entrainment tube	Spray freeze–dried chitosan nanoparticles containing pDNA	Fine particle fraction of 20% and MMAD 1.7 micron was reported from NGI studies The dry powder of pDNA was shown to maintain 80–90% of its efficacy for 3 months without the requirement for ultra-cold storage conditions
Cheow et al. 2011 [106]	Standardized powder entrainment tube	Dry powders of poly(caprolactone) nanoparticles encapsulating levofloxacin using polyvinyl alcohol (PVA) and mannitol as excipients	In vitro emitted dose of 90% and FPF of 14% from NGI studies reported

Table II (continued)

Name and year of study	Type of inhaler/device used	Formulation discussed	In vitro parameters and/or in vivo efficacy reported
Fiedler et al. (2021) [107]	Not applicable	Spray-dried interleukin-8 using phosphate-buffered saline as the dissolution media	Dry powder versions of interleukin were shown to be stable after 12 weeks of storage without any signs of protein unfolding, compromised binding affinity
Lo et al. 2021 [108]	Size 3 gelatin capsule (Capsugel®, Morristown, NJ, USA) was loaded into Breezhaler® (Novartis AG, Basel, Switzerland)	Spray freeze–dried bovine serum albumin using hydroxypropyl beta-cyclodextrin as the protein stabilizer	FPF in the range of 60 to 80% and MMAD in the range of 1–5 micron were reported for the tested formulations based on NGI studies
Liang et al. (2018) [109]	Breezhaler® (Novartis AG, Basel, Switzerland)	Spray freeze–dried powders of DNA and siRNA	FPF of 28% from NGI studies were reported along with intact structure of siRNA post spray drying
Otake et al. (2016) [110]	No. 2 HPMC capsule (Shionogi Qualicaps Co., Ltd., Tokyo, Japan), fitted into Jethaler®, Hitachi Automotive Systems, Co., Ltd	Spray freeze–dried powders of amino acids	More than 50% FPF was reported for most of the dry powder versions of the amino acid when tested using the twin-stage liquid impinger (TSLI)
Milani et al. (2020) [111]	Size 2 HPMC capsules placed in a Cyclohaler®	Spray freeze–dried human IgG antibody using hydroxypropyl beta-cyclodextrin as the protein stabilizer	FPF in the range of 48–50% were obtained for the IgG dry powders using the impinger
Pouya et al. (2018) [121]	Size 2 HPMC capsules placed in a Cyclohaler®	Spray freeze–dried human IgG antibody using hydroxypropyl beta-cyclodextrin as the protein stabilizer along with trehalose	FPF of about 50% were obtained for the IgG dry powders using the impinger
Liang et al. (2014) [112]	Size 3 hydroxypropyl methylcellulose capsules (Capsugel, West Ryde, NSW, Australia) placed into a dry powder inhaler (Osmohaler™, Plastiape, Osnago, Italy)	Dry powder formulations of pH responsive peptides and plasmid DNA, with mannitol as carrier	FPF of about 50% was reported for the dry powders using the twin-stage impinger Successful transfection of the A549 cells by the dry powder DNA particles was also reported
Liang et al. (2015) [113]	Size 3 hydroxypropyl methylcellulose capsules (Capsugel, West Ryde, NSW, Australia) placed into the Aerolizer™	Dry powder formulations of pH responsive peptides and siRNA, with mannitol as carrier	FPF of about 40% was reported for the dry powders using the twin-stage impinger Successful transfection of the A549 cells by the dry powder DNA particles was also reported
Chow et al. (2017) [114]	Size 3 hydroxypropyl methylcellulose capsule (Capsugel, West Ryde, NSW, Australia), which was placed in a Breezhaler® (Novartis Pharmaceuticals, Hong Kong)	siRNA co-spray dried with mannitol and leucine	FPF of 45% by incorporating mannitol and leucine in the formulation was reported from NGI studies Structural integrity of the siRNA was shown to be intact post spray drying based on gel retardation assay
Okuda et al. (2013) [115]	N/A	Dry powders of siRNA containing chitosan and mannitol prepared using supercritical fluid carbon dioxide precipitation	The dry powder siRNA particles were shown to successfully transfect in lung epithelium, when intra-tracheally administered to mice
Todo et al. (2002) [116]	An hydroxypropyl methylcellulose (HPMC) capsule (size 2, Shionogi Qualicaps Company, Nara, Japan) loaded in an inhaler Jethaler (Hitachi Unisia Automotive, Ltd., Atsugi, Japan)	Dry powder of bovine insulin prepared by spray drying and supercritical carbon dioxide–based precipitation technique using mannitol as the carrier	In vitro cascade impaction studies resulted in MMAD of 3.2 microns and FPF of 48% In vivo efficacy of intra-tracheally delivered dry powder insulin was also demonstrated via rapid decline in blood glucose levels post dosing to rats

Author Contribution Sayeed Mossadeq is the first and primary author of this review article and has made the entire contributions including the entire conception of the work and drafting of the work for important intellectual content along with approving final version to be published. Other co-authors like Dr. Rajen Shah, Viraj Shah, and Dr. Milind Bagul have reviewed the content of the review article, evaluated it for its scientific validity along with making suggestions to revise certain areas.

Declarations

Conflict of Interest The authors declare no competing interests.

References

1. https://www.who.int/emergencies/diseases/novel-coronavirus-2019 - accessed December 3rd 2021
2. Russell, et al. Mucosal immunity in COVID-19: a neglected but critical aspect of SARS-CoV-2 infection. Front Immunol. 2020;11:611337.
3. Gallo, et al. The central role of the nasal microenvironment in the transmission, modulation, and clinical progression of SARSCoV-2 infection. Mucosal Immunol. 2020;14:305–16.
4. Shang, et al. Cell entry mechanisms of SARS-CoV-2. PNAS. 2020;117(21):11727–34.
5. Hamming I, et al. Tissue distribution of ACE2 protein, the functional receptor for SARS coronavirus. A first step in understanding SARS pathogenesis. J Pathol. 2004;203:631–7.
6. Samavati, et al. ACE2, Much more than just a receptor for SARS-CoV-2. Front Cell Infect Microbiol. 2020;10:317.
7. Lei, et al. SARS-CoV-2 spike protein impairs endothelial function via down regulation of ACE2. Circ Res. 2021;128:1323–6.
8. Li G, et al. Assessing ACE2 expression patterns in lung tissues in the pathogenesis of COVID-19. J Autoimmun. 2020;112:102463.
9. Sungnak W, et al. SARS-CoV-2 entry factors are highly expressed in nasal epithelial cells together with innate immune genes. Nat Med. 2020;26:681–7.
10. Batah, et al. Pulmonary pathology of ARDS in COVID-19: a pathological review for clinicians. Respir Med. 2021;176:106239.
11. Zhou F, et al. Clinical course and risk factors for mortality of adult inpatients with COVID-19 in Wuhan, China: a retrospective cohort study. The Lancet. 2020;395:1054–62.
12. Eedara, et al. Inhalation delivery for the treatment and prevention of COVID-19 infection. Pharmaceutics. 2021;13(7):1077. https://doi.org/10.3390/pharmaceutics13071077.
13. Mitchell, et al. Urgent appeal from International Society for Aerosols in Medicine (ISAM) during COVID-19: clinicaldecision makers and governmental agencies should consider the inhaled route of administration: a statement from the ISAM Regulatory and Standardization Issues Networking Group. J Aerosol Med Pulm Drug Deliv. 2020;33(4):235–8. https://doi.org/10.1089/jamp.2020.1622.
14. Baden, et al. Efficacy and safety of the mRNA-1273 SARS-CoV-2 vaccine. New England J Med. 2021;384:403-416. https://doi.org/10.1056/NEJMoa2035389.
15. Thomas, et al. Six month safety and efficacy of the BNT162b2 mRNA COVID-19 vaccine. medRxiv preprint. 2021. https://doi.org/10.1101/2021.07.28.21261159.
16. Wheeler, et al. Differential antibody response to mRNA COVID-19 vaccines in healthy subjects. Microbiology Spectrum. Am Soc Microbiol. 2021;9(1):e00341–21.
17. Jalkanen, et al. COVID-19 mRNA vaccine induced antibody responses against three SARS-CoV-2 variants. Nat Commun. 2021;12:3991. https://doi.org/10.1038/s41467-021-24285-4.
18. Campillo-Luna J, et al. Human IgG and IgA responses to COVID-19 mRNA vaccines. medRxiv. PLOS ONE. 2021. https://doi.org/10.1371/journal.pone.0249499.
19. Schroeder, et al. Structure and function of immunoglobulins. J Allergy Clin Immunol. 2010;125(202):S41-S52. https://doi.org/10.1016/j.jaci.2009.09.046.
20. Horton, et al. Antibodies and their receptors: different potential roles in mucosal defense. Front Immunol. 2013;4. https://doi.org/10.3389/fimmu.2013.00200.
21. Sterlin, et al. IgA dominates the early neutralizing antibody response to SARS-CoV-2. Sci Transl Med. 2021;13.
22. Boehm MK, et al. The fab and fc fragments of IgA1 exhibit a different arrangement from that in IgG: a study by X-ray and neutron solution scattering and homology modeling. J Mol Biol. 1999;286(5):1421–47.
23. Wec AZ, et al. Broad neutralization of SARS-related viruses by human monoclonal antibodies. Science. 2020;369:731–6.
24. Wang, et al. Enhanced SARS-CoV-2 neutralization by dimeric IgA. Sci Transl Med. 2021;13(577):eabf1555.
25. Afkhami S, et al. Respiratory mucosal delivery of next-generation COVID-19 vaccine provides robust protection against both ancestral and variant strains of SARS-CoV-2. Cell. 2022;185:896–915.
26. https://www.imperial.ac.uk/news/203653/landmark-coronavirus-study-trial-inhaled-imperial/. Accessed 12 Dec 2021.
27. Chavda, et al. Intranasal vaccines for SARS-CoV-2: from challenges to potential in COVID-19 management. Drug Discov Today. 2021;26(11):2619–36. https://doi.org/10.1016/j.drudis.2021.07.021.
28. Horby, et al. Dexamethasone in hospitalized patients with COVID-19. N Engl J Med. 2021;384:693-704. https://doi.org/10.1056/NEJMoa2021436
29. Ramakrishnan S, et al. Inhaled Budesonide in the treatment of early COVID-19 (STOIC): a phase 2, open label, randomized controlled trial. Lancet Respir Med. 2021;9(7):763–72. https://doi.org/10.1016/S2213-2600(21)00160-0.
30. Yu, et al. Inhaled budesonide from COVID-19 in people at high risk of complications in the community in the UK(PRINCIPLE): a randomized, controlled, open-label, adaptive platform trial. The Lancet. 2021;398(10303):843–5. https://doi.org/10.1016/S0140-6736(21)01744-X.
31. Iwabuchi K, et al. Therapeutic potential of ciclesonide inhalation for COVID-19 pneumonia: Report of three cases. J Infect Chemother. 2020;26:625–32.
32. Clemency, et al. Efficacy of inhaled ciclesonide for outpatient treatment of adolescents and adults with symptomatic COVID-19, a randomized clinical trial. JAMA Int Med. 2022;182(1):42–9. https://doi.org/10.1001/jamainternmed.2021.6759.
33. Berlin, et al. Severe Covid-19 2021. N Engl J Med. 2021;383:252021. https://doi.org/10.1056/NEJMcp2009575.
34. Fazzini, et al. Effectiveness of prone position in spontaneously breathing patients with COVID-19: a prospective cohort study. J Intensive Care Soc. 2021;1(1):1–4. https://doi.org/10.1177/1751143721996542.
35. Dey J, et al. Neuroinvasion of SARS-CoV-2 may play a role in the breakdown of the respiratory center of the brain. J Med Virol. 2020;93:1296–303.
36. Satturwar, et al. Postmortem findings associated with SARS-CoV-2., systematic review and meta analysis. Am J Surg Pathol. 2021;45(5):587–603. https://doi.org/10.1097/PAS.0000000000001650.
37. Menter, et al. Postmortem examination of COVID-19 patients reveals diffuse alveolar damage with severe capillary congestion and variegated findings in the lungs and other organs suggesting vascular dysfunction. Histopathology. 2020;77 (198–209)(2): https://doi.org/10.1111/his.14134.

38. Meinhardt J, *et al*. 2021 Olfactory transmucosal SARS-CoV-2 invasion as a portal of central nervous system entry in individuals with COVID-19. Nat Neurosci. 2021;24:168–75.

39. Bennett G, *et al*. *In vitro* study of the effect of breathing pattern on aerosol delivery during high flow nasal therapy. Pulm Ther. 2019;5:43–5.

40. Kondo T, *et al*. Appropriate use of a dry powder inhaler based on inhalation flow pattern. J Pharm Health Care Sci. 2017;3:5.

41. Ramadan W, *et al*. Patterns of use of dry powder inhalers *versus* pressurized metered dose inhaler devices in adult patients with chronic obstructive pulmonary disease or asthma: An observational comparative study. Chronic Respir Dis. 2017;14(3):309–20.

42. Melchor R, *et al*. Lung deposition patterns of directly labeled salbutamol in normal subjects and in patients with reversible airflow obstruction. Thorax. 1993;48:506–11.

43. McCabe, et al. ProAir HFA delivers warmer, lower impact, longer duration plumes containing higher fine particle dose than Ventolin HFA. J Aerosol Med Pulm Drug Deliv. 2021;25(2):104–9. https://doi.org/10.1089/jamp.2011.0891.

44. Kunda, et al. A novel approach study the pMDI plume using an infrared camera and to evaluate the aerodynamic properties after varying the time between actuations. Int J Pharm. 2017;526(1-2):41–9. https://doi.org/10.1016/j.ijpharm.2017.04.051.

45. Begat P, Price R. The influence of force control agents on the cohesive-adhesive balance in dry powder inhaler formulations. KONA. 2005;23(23):109–21. https://doi.org/10.14356/kona.2005014.

46. Duke, et al. Increasing the fine particle fraction of pressurized metered dose inhaler solutions with novel actuator shapes. Int J Pharm. 2021;597:120341. https://doi.org/10.1016/j.ijpharm.2021.120341.

47. Demoly P, *et al*. The clinical relevance of dry powder inhaler performance for drug delivery. Respir Med. 2014;108:1195–203.

48. Ibrahim M, *et al*. Inhalation drug delivery devices: technology update. Med Devices: Evid Res. 2015;8:131–9.

49. Longest W, *et al*. Devices for improved delivery of the nebulized pharmaceutical aerosols to the lungs. J Aerosol Med Pulm Drug Deliv. 2019;32(5):317–39.

50. Assessment report for COVID-19 Vaccine Moderna from European Medicines Agency. Procedure number EMEA/H/C/005791/000. 2021, EMA/15689/2021.

51. Background document on the mRNA-1273 vaccine (Moderna) against COVID-19. World Health Organization (WHO). 2021. WHO Reference number: WHO/2019-nCoV/vaccines/SAGE-recommendation/mRNA-1273/background/2021.1.

52. FDA Briefing Document titled EUA amendment request for a booster dose of the Moderna COVID-19 Vaccine. Vaccines and related biological products advisory committee meeting. 2021. https://www.fda.gov/media/152991/download.

53. FDA factsheet for healthcare providers administering vaccine (vaccination providers) titled EUA of The Moderna COVID-19 vaccine to prevent COVID-19. 2021. https://eua.modernatx.com/covid19vaccine-eua/eua-fact-sheet-providers.pdf?.

54. Schoenmaker, et al. mRNA-lipid nanoparticle COVID-19 vaccines: structure and stability. Int J Pharm. 2021;601:120586. https://doi.org/10.1016/j.ijpharm.2021.120586.

55. Pfizer-BioNTech COVID-19 vaccine. FDA briefing document. Vaccines and related biological products advisory committee meeting. 2020. https://www.fda.gov/media/159195/download.

56. Teo, et al. Review of COVID-19 mRNA vaccines: BNT162b2 and mRNA-1273. J Pharm Pract. 2021;12:8971900211009650. https://doi.org/10.1177/08971900211009650.

57. COMIRNATY. European Medicines Agency, Assessment report. EMA/CHMP/282047/2021 Rev. 1, 22 July 2021 and Assessment report. EMEA/H/C/005735/000. EMA/CHMP/282047/2021

Rev.1 Committee for Medicinal Products for Human Use (CHMP). 2021. https://www.ema.europa.eu/en/documents/variation-report/comirnaty-h-c-5735-ii-0030-epar-assessmentreport-variation_en.pdf.

58. Crommelin, et al. Addressing the cold reality of mRNA vaccine stability. J Pharm Sci. 2020;110(3):997–1001. https://doi.org/10.1016/j.xphs.2020.12.006.

59. Sheth, et al. Factors influencing aerodynamic particle size distribution of suspension pressurized metered dose inhalers. AAPS PharmsciTech. 2015;16(1):192–201. https://doi.org/10.1208/s12249-014-0210-z.

60. Zhu, et al. Aerosol particle generation from solution-based pressurized metered dose inhalers: a technical overview of parameters that influence respiratory disease. Pharm Dev Technol. 2014;20(8):1–14. https://doi.org/10.3109/10837450.2014.959176.

61. Wallington, et al. The environmental impact of CFC replacements – HFCs and HCFCs. Environ Sci Technol. 1994;28(7):320A–6A. https://doi.org/10.1021/es00056a714.

62. United Nations Environment Program website. Available at https://www.unep.org/news-and-stories/story/montreal-protocol-triumph-treaty., accessed December 16 2021

63. Pritchard, et al. The climate is changing for metered-dose inhalers and action is needed. Drug Des Dev Ther. 2020;14:3043–55. https://doi.org/10.2147/DDDT.S262141.

64. Market characterization of the U.S. metered dose inhaler industry. United States environmental protection agency., Stratospheric protection division. Office of air and radiation., Washington., D.C. 20460., September 2021. Accessible at https://www.epa.gov/sites/default/files/2021-03/documents/epa-hq-oar-2021-0044-0002_attachment_1-mdis.pdf

65. Janson, et al. Carbon footprint impact of the choice of inhalers for asthma and COPD. Thorax. 2019;75(1):82–4. https://doi.org/10.1136/thoraxjnl-2019-213744.

66. Lavorini, et al. Retail sales of inhalation devices in European countries: so much for a global policy. Respir Med. 2011;105(7):1099–1103. https://doi.org/10.1016/j.rmed.2011.03.012.

67. Boyd G. The continued need for metered dose inhalers. J Aerosol Med. 1995;8(1):S-9-S−12.

68. Myrdal, et al. Advances in metered dose inhaler technology: formulation development. AAPS Pharmsci Tech. 2014;15(2):434–55. https://doi.org/10.1208/s12249-013-0063-x.

69. Gavtash B, *et al*. Transient aerodynamic atomization model to predict aerosol droplet size of pressurized metered dose inhalers (pMDI). Aerosol Sci Technol. 2017;51(8):998–1008.

70. Conti DS, *et al*. Propellant-based inhalers for the non-invasive delivery of genes via oral inhalation. J Control Release. 2012;157:406–17 (**Available online 1 October 2011**).

71. Bains BK, *et al*. *In vitro* reporter gene transfection via plasmid DNA delivered by metered dose inhaler. J Pharm Sci. 2010;99(7):3089–99.

72. Li Y, *et al*. Novel pMDI formulations for pulmonary delivery of proteins. Int J Pharm. 2010;385:73–8.

73. Quinn EA, *et al*. Protein conformational stability in the hydrofluoroalkane propellants tetrafluoroethane and heptafluoropropane analyzed by Fourier transform Raman spectroscopy. Int J Pharm. 1999;186:31–41.

74. Oliver, et al. Initial assessment of a protein formulated in pressurized MDIs for pulmonary delivery. RDD VII. 2000;2:279–82.

75. Brown, et al. Propellant-driven aerosols of proteins. Aerosol Sci Technol. 1996;24(1):45–56. https://doi.org/10.1080/02786829608965351.

76. Williams III, et al. Formulation of a protein with propellant HFA 134a for aerosol delivery. Eur J Pharm Sci. 1998;7(2):37–144. https://doi.org/10.1016/S0928-0987(98)00015-3.

77. Nakate T, *et al*. Comparison of the lung absorption of FK224 inhaled from a pressurized metered dose inhaler and a dry powder inhaler by healthy volunteers. Eur J Pharm Biopharm. 2003;56:319–25.

78. Nyambura BK, *et al*. The processing of nanoparticles containing proteins for suspension in hydrofluoroalkane propellants. Pharm Nanotechnol. 2009;372:140–6.

79. Hausmann M, *et al*. Inhaled insulin as adjunctive therapy in subjects with type 2 diabetes failing oral agents: a controlled proof-of-concept study., Diabetes. Obes Metab. 2006;8:574–80.

80. Adjei, et al. Bioavailability of Leuprolide Acetate following nasal and inhalation delivery to rats and healthy humans. Pharm Res. 1992;9(2):244–9. https://doi.org/10.1023/a:1018997625726.

81. Vyas SP, *et al*. Pressurized pack based liposomes for pulmonary targeting of isoprenaline – development and characterization. J Microencapsul. 1994;11(4):373–80.

82. Farr SJ, *et al*. Assessing the potential of aerosol-generated liposomes from pressurized pack formulations. J Control Release. 1985;5:119–27.

83. Bharatwaj B, *et al*. The potential for the noninvasive delivery of polymeric nanocarriers using propellant-based inhalers in the treatment of Chlamydial respiratory infections. Biomaterials. 2010;31:7376–85.

84. Uddin N, *et al*. Challenges of storage and stability of mRNA-based COVID-19 vaccines. Vaccines. 2021;9:1033.

85. Byron PR. Determinants of drug and polypeptide bioavailability from aerosols delivered to the lungs. Adv Drug Deliv Rev. 1990;5:107–32.

86. Lambert D, *et al*. Denaturation of RNA secondary and tertiary structure by urea:simple unfolded state models and free energy parameters account for measured m-values. Biochemistry. 2012;51(44):9014–26.

87. Malberg, et al. Physiological respiratory parameters in pre-hospital patients with suspected COVID-19: a prospective cohort study. PLOS ONE. 2021;16(9):e0257018. https://doi.org/10.1371/journal.pone.0257018.

88. Grasselli G, *et al*. Mechanical ventilation parameters in critically ill COVID-19 patients: a scoping review. Critical Care. 2021;25:115.

89. Pan, et al. Airway resistance variation correlates with prognosis of critically ill COVID-19 patients: a computational fluid dynamics study. Comp Methods Prog Biomed. 2021;208:106257. https://doi.org/10.1016/j.cmpb.2021.106257.

90. Darquenne C. Aerosol deposition in health and disease. J Aerosol Med Pulm Drug Deliv. 2012;25(3):140–7. https://doi.org/10.1089/jamp.2011.0916.

91. Cheng, et al. Mechanisms of pharmaceutical aerosol deposition in the respiratory tract. AAPS PharmSciTech. 2014;15(3):630–40. https://doi.org/10.1208/s12249-014-0092-0.

92. Brown, et al. The stability of a particle size reduced therapeutic protein in an HFA MDI formulation. Respiratory Drug Delivery VIII. 2002;2:311–4.

93. Kapitza, et al. Time action profile of a new pulmonary insulin applied with a metered-dose inhaler. Diabetes. 2003;52(suppl1):A91.

94. Frijlink, et al. Dry powder inhalers for pulmonary drug delivery. Exp Opin Drug Del. 2004;1(1):67–86. https://doi.org/10.1517/17425247.1.1.67.

95. Shoyele SA, *et al*. Prospects of formulating proteins/peptides as aerosols for pulmonary drug delivery. Int J Pharm. 2006;314:1–8.

96. Qiu Y, *et al*. Effective mRNA pulmonary delivery by dry powder formulation of PEGylated synthetic KL4 peptide. J Control Release. 2019;314:102–15.

97. Emig, et al. AUG-3387, A human-derived monoclonal antibody neutralizes SARS-CoV-2 variants and reduces viral load from therapeutic treatment of hamsters in vivo. bioRxiv. 2021. https://doi.org/10.1101/2021.10.12.464150.

98. Audouy, et al. Development of a dried influenza whole inactivated virus vaccine for pulmonary immunization. Vaccine. 2011;29(26):4345–52. https://doi.org/10.1016/j.vaccine.2011.04.029.

99. Patil, et al. Evaluation of a monophosphoryl lipid A as adjuvant for pulmonary delivered influenza vaccine. J Control Release. 2014;174:51–62. https://doi.org/10.1016/j.jconrel.2013.11.013.

100. Amorij J-P, *et al*. Pulmonary delivery of an inulin-stabilized influenza subunit vaccine prepared by spray-freeze drying induces systemic, mucosal humoral as well as cell-mediated immune responses in BALB/c mice. Vaccine. 2007;25:8707–17.

101. Smith DJ, *et al*. Evaluation of novel aerosol formulations designed for mucosal vaccination against influenza virus. Vaccine. 2003;21:2805–12.

102. Lin WH, *et al*. Successful respiratory immunization with dry powder live-attenuated measles virus vaccine in rhesus macaques. PNAS. 2011;108(7):2987–92.

103. Burger JL, *et al*. Stabilizing formulations for inhalable powders of live-attenuated measles virus vaccine. J Aerosol Med Pulm Drug Deliv. 2008;21(1):25–34.

104. Agarkhedkar S, *et al*. Safety and immunogenicity of dry powder measles vaccine administered by inhalation: a randomized controlled phase I clinical trials. Vaccine. 2014;32:6791–7.

105. Tran T-T, *et al*. Effects of storage on the stability and aerosolization efficiency of dry powder inhaler formulation of plasmid DNA-chitosan nanoparticles. J Drug Deliv Sci Technol. 2020;59:101866.

106. Cheow WS, *et al*. Spray-freeze-drying production of thermally sensitive polymeric nanoparticle aggregates for inhaled drug delivery: effect of freeze-drying adjuvants. Int J Pharm. 2011;404:289–300.

107. Fiedler D, *et al*. Comparing freeze drying and spray drying of interleukins using model protein CXCL8 and its variants. Eur J Pharm Biopharm. 2021;168:152–65.

108. Lo JCK, *et al*. Inhalable protein powder prepared by spray-freeze-drying using hydroxypropyl-β-cyclodextrin as excipient. Pharmceutics. 2021;13:615.

109. Liang W, *et al*. Spray freeze drying of small nucleic acids as inhaled powder for pulmonary delivery. Asian J Pharm Sci. 2018;13:163–72.

110. Otake H, *et al*. Development of spray-freeze-dried powders for inhalation with high inhalation performance and antihygroscopic property. Chem Pharm Bull. 2016;64(3):239–45.

111. Milani, et al. Hydroxypropyl beta cyclodextrin: a water-replacement agent or a surfactant upon spray freeze-drying of IgG with enhanced stability and aerosolization. Drug Dev Ind Pharm. 2020;46(3):403–11. https://doi.org/10.1080/03639045.2020.1724131.

112. Liang W, *et al*. Formulation of pH responsive peptides as inhalable dry powders for pulmonary delivery of nucleic acids. Eur J Pharm Biopharm. 2014;86:64–73.

113. Liang, et al. Inhalable dry powder formulations of siRNA and pH responsive peptides with antiviral activity against H1N1 influenza virus. Mol Pharm. 2015;12(3):910–21. https://doi.org/10.1021/mp500745v.

114. Chow, et al. Inhaled powder formulations of naked siRNA using spray drying technology with L-leucine as dispersion enhancer. Int J Pharm. 2017;530(1-2):40–52. https://doi.org/10.1016/j.ijpharm.2017.07.013.

115. Okuda T, *et al*. Gene silencing in a mouse lung metastasis model by an inhalable dry small interfering RNA powder prepared using the supercritical carbon dioxide technique. Biol Pharm Bull. 2013;36(7):183–1191.

116. Todo, et al. Improvement of insulin absorption from intratracheally administered dry powder prepared by supercritical carbon dioxide process. J Pharm Sci. 2003;92(12):2475–86. https://doi.org/10.1002/jps.10497.

117. Okamoto H, et al. Chitosan-interferon-β gene complex powder for inhalation treatment of lung metastasis in mice. J Control Release. 2011;150:187–95.

118. World Health Organization (WHO) Living guidelines for clinical management of COVID-19. WHO Reference number: WHO/2019-nCoV/clinical/2021.2. 2021. https://www.who.int/publications/i/item/WHO-2019-nCoV-clinical-2021-2.

119. Capstick TGD, *et al*. Inhaler technique and training in people with chronic obstructive pulmonary disease and asthma. Exp Rev Respir Med. 2012;6(1):91–103.

120. Jogi R, *et al*. Inspiratory flow parameters through dry powder inhalers in healthy volunteers and patients with chronic obstructive pulmonary disease (COPD): device resistance does not limit use in COPD. Int J Chron Obstruct Pulmon Dis. 2021;16:1193–201.

121. Pouya MA, *et al*. Spray-freeze drying: a suitable method for aerosol delivery of antibodies in the presence of trehalose and cyclodextrins. AAPSPharmSciTech. 2018;19:2247–54.