Expert Opinion

Recent advances in liposomal dry powder formulations: preparation and evaluation

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Liposomal drug dry powder formulations have shown many promising features for pulmonary drug administration, such as selective localization of drug within the lung, controlled drug release, reduced local and systemic toxicities, propellant-free nature, patient compliance, high dose carrying capacity, stability and patent protection. Critical review of the recent developments will provide a balanced view on benefits of liposomal encapsulation while developing dry powder formulations and will help researchers to update themselves and focus their research in more relevant areas. In liposomal dry powder formulations (LDPF), drug encapsulated liposomes are homogenized, dispersed into the carrier and converted into dry powder form by using freeze drying, spray drying and spray freeze drying. Alternatively, LDPF can also be formulated by supercritical fluid technologies. On inhalation with a suitable inhalation device, drug encapsulated liposomes get rehydrated in the lung and release the drug over a period of time. The prepared LDPF are evaluated *in vitro* and *in vivo* for lung deposition behavior and drug disposition in the lung using a suitable inhaler device. The most commonly used liposomes are composed of lung surfactants and synthetic lipids. Delivery of anticancer agents for lung cancer, corticosteroids for asthma, immunosuppressants for avoiding lung transplantation rejection, antifungal drugs for lung fungal infections, antibiotics for local pulmonary infections and cystic fibrosis and opioid analgesics for pain management using liposome technology are a few examples. Many liposomal formulations have reached the stage of clinical trials for the treatment of pulmonary distress, cystic fibrosis, lung fungal infection and lung cancer. These formulations have given very promising results in both *in vitro* and *in vivo* studies. However, modifications to new therapies for respiratory diseases and systemic delivery will provide new challenges in conducting well-designed inhalation toxicology studies to support these products, especially for chronic diseases.

Keywords: dry powder formulations, inhalers, liposomes, pulmonary

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1. Introduction

Advances in drug formulation and inhalation device design are creating new opportunities for inhaled drug delivery as an alternative to oral and parenteral delivery methods. Growing attention has been given to the potential of the pulmonary route as a non-invasive administration route for local and systemic delivery of potent therapeutics, because pulmonary delivery could provide rapid onset of action, high pulmonary bioavailability, thin (0.1 – 0.2 μm) alveolar epithelium and large absorptive surface area (up to 100 m^2) for local drug action, rapid systemic drug absorption, absence of first-pass metabolism and high

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perfusion of blood supply [1,2]. Despite these advantages and the widespread use of therapeutics in the lung, there are several shortcomings associated with drug delivery to the respiratory tract. Although the onset of action is very rapid, the duration is often short-lived as the drug can be quickly removed from the lung through various clearance mechanisms [3,4]. Regardless of the type of aerosol device employed, most patients require dosing every 4 – 6 h, and more frequently in some instances; both dose and frequency of dosing changes with time.

Inhaled drug delivery systems can be divided into three principal categories as pressurized metered-dose inhalers (pMDIs), nebulizers and dry powder inhalers (DPIs). pMDIs are most popular method for pulmonary drug delivery. Typically, a pMDI formulation contains an active ingredient, generally in solution or suspension, along with inactive excipients. These systems are tamper-proof and deliver an accurate and reproducible dose of aerosolized drugs to the lung, but they suffer from the limitation of problems associated with chlorofluorocarbon-based propellants, low lung deposition and high oropharyngeal deposition resulting from high velocity delivery. However, with the evolution of hydrofluroalkane-based pMDIs and the development of new designs at the drug and device level, the pMDIs have gone through a process of revival within the last five years, with encouraging results [5].

Apart from pMDIs, nebulizers and DPIs are widely used as alternative propellant-free devices for pulmonary delivery. Nebulizers use ultrasound or compressed gas to produce aerosol droplets in the respirable size range from aqueous solutions of drugs containing cosolvents and pharmaceutical aids to ensure the physical and chemical stability of drug. They are widely used to deliver β2 agonists, corticosteroids, anti-allergics, anti-cholinergics, antibiotics, mucolytics and other therapeutic agents to the respiratory tract [6]. Nebulizers may be inhaled during normal tidal breathing through a mouthpiece or facemask and can be used to deliver aerosolized drugs to children, the elderly and patients with arthritis, and are frequently used for drugs with a high therapeutic dose [7]. Although nebulizers can virtually nebulize any drug into the lung, they suffer from the limitations of high drug loss during inhalation, with only about 10 – 30% of drug reaching the lung [8]. The quantity of the drug reaching the lung is a characteristic of the type of nebulizer used for the study and may vary from 10 – 50% for the same sample [9]. However, the dose variation may be important for the drugs with dose-related side effects, such as steroids, and for expensive medications as rhDNase [10]. Nebulizers vary greatly in the size of droplet they produce, their nebulization time and drug output by up to 100%. They are also time-consuming to deliver, bulky, non-portable, with relatively with high cost expenses. However, the various limitations of the jet nebulizers have been resolved with new technological advances and improved nebulizer designs. Newer devices employed are vibrating mesh or aperture plate (VM/AP) for the generation of therapeutic aerosols, and these were found to produce consistent aerosols with increased efficiency, predominant aerosol fine particle fractions (FPF), low residuals and the ability to nebulize even microliter volumes. Current VM/AP devices in clinical use are the Omron MicroAir (OMRON Healthcare Europe B.V., UK), the Nektar Aeroneb (Aeroneb, USA) and the Pari eFlow (Pari Pharma GmbH, Germany). However, some devices are only approved for use with specific medications [11]. Another device, a Respimat Soft Mist Inhaler have been developed, which is a multidose, hand-held, liquid inhaler that results in the generation of a higher fraction of fine particles [12].

DPIs are devices for delivering a dry powder formulation of an active drug for local or systemic effect by a suitable inhaler device. The development of DPIs has been motivated by the desire for alternatives to pMDIs and nebulizers to overcome their disadvantages and to facilitate the delivery of macromolecules and products of biotechnology. In general DPIs are easier to use, more stable and efficient systems. Since DPIs are typically formulated as one-phase, solid particle blends, they are also preferred from a stability and processing standpoint [13]. Unlike pMDIs, DPIs avoid the problems inherent in the use of propellant gases and the need for coordination of inhalation and actuation [14]. DPIs are also very portable, patient-friendly, easy to use and do not require spacers [15]. The DPIs are compact in nature, breath actuated, easy to use, with high drug dose carrying capacities, high lung deposition (from 50 – 70%) and minimal extra pulmonary loss of drug due to low oropharyngeal deposition, low device retention and low exhaled loss. DPIs are subject to strict pharmaceutical and manufacturing standards by regulatory bodies, the most challenging of which is the demonstration of device reliability in terms of delivered dose uniformity [16]. Apart from formulation technology the inhaler device plays an important role in pulmonary delivery of DPIs. Inhaler devices also suffer from some inherent limitations which need to be taken into consideration for the optimum delivery of formulation to lungs.

Pulmonary disposition of drugs is associated with lung clearance mechanisms operating with high reticulo-endothelial system (RES) uptake, irrespective of the method used for delivery. In consequence, this drawback leads to high drug dosing frequency, causing patient incompliance (4 – 6 times in some diseases) and increased dose with time. Although conventional DPIs were found to be more useful for enhanced pulmonary delivery, there was a need for more efficient and controlled delivery to the lungs via DPIs for enhanced efficacy of the drug by increasing pulmonary residence time and reducing lung clearance.

Liposomes, a phospholipid bilayer system enclosing aqueous compartments, provide an efficient delivery system for the treatment of pulmonary disorders because they are

biocompatible, biodegradable and relatively non-toxic [17]. Liposomes can significantly alter the pharmacokinetics and pharmacodynamics of entrapped drugs [18,19]. Targeting drug delivery into the lungs has become one of the most important aspects of systemic or local drug delivery systems. To convey a sufficient dose of drug to the lungs, suitable drug carriers are required. Administration of liposomes to the respiratory tract is particularly attractive because of the accessibility of the lung as a local target organ, the compatibility of liposomes and lung surfactant components (85% phospholipid), the need for sustained local therapy following inhalation [20], the improved therapeutic index of the drug due to enhanced intracellular delivery and slow systemic dilution and clearance, minimizing/eliminating side effects, reducing dose/frequency of dosing and possibly drug resistance, systemic toxicities and the cost of therapy [18,21,22].

Studies have indicated that liposomes can be effectively deposited in the human respiratory tract by aerosolization. Delivery of liposomes in suspension form has been investigated by various researchers using either a nebulizer or pMDIs and is under clinical trials. However, in a dispersed aqueous system, liposomes have problems associated with lipid degradation by hydrolysis or oxidation and sedimentation, aggregation, leakage of drugs or fusion of liposomes during storage [23]. The liposomal suspension during storage and aerosolization may result in inadequate chemical and physical stability. Physical and chemical stability of liposome during aerosolization from suspension form is also another concern, which needs to be addressed in the delivery of liposome suspension to lungs. Although instable in suspension form, the liposomes processed using freeze drying, spray drying, spray freeze drying or supercritical fluid technology into dry powder form help to achieve long-term stability and overcome the issues of degradation by hydrolysis or oxidation and sedimentation, drug leakage, aggregation, or fusion of liposomes associated with liposome in suspension form [1,22]. The dry liposomes encapsulating therapeutics can be delivered to lungs as a DPI, due to their propellant free nature, high stability, flexibility in formulation development and patient compliance. However, the performance of DPIs relies on many aspects including the design and type of inhaler device, the powder formulation and the airflow generated by the patient [24-26]. This article critically reviews the recent literature pertaining to the pulmonary delivery of liposomal DPIs and evaluates the present and future trends in order to help researchers to select their research goals with better understanding.

2. Preparation methods for LDPF

Recently, lipid-based particulate systems have been extensively used in pulmonary drug delivery for their ability to control the drug release properties with their biocompatibility, biodegradability and non-immunogenicity [17]. The most commonly used lipid-based systems include liposomes (lipid bilayers encapsulating water), proliposomes (dry liposomes which form liposomes ready for use when they come into contact with water) [27], cochleates and nanocochleates (the precipitate formed by treating liposomes containing two-phase polymer solution, with positively charged molecules such as Ca^{2+} or Zn^{2+}) [28], *in situ* liposomes from a lipid matrix and drug with varying transition temperature in dry form (55 – 60°C) and hydrated form (37°C) [29], large respirable particles (particles with a geometric diameter above 5 µm and density below 0.4 g/cm^3) [30] and light porous particles [31]. However, of all these lipid-based systems, liposomes are still the most widely researched particulate system because of their simplicity of formation and high formulation variability to serve the need of the formulation.

Many methods have so far been reported for the preparation of liposomes, such as the Bangham method, the organic solvent injection method and the reverse phase evaporation method, among others. Liposomes encapsulating the drugs can be prepared by any of the techniques reported in the literature, based upon the desired characteristics of liposomes, which can be later processed in a dry powder form for inhalation. Recently, liposomes prepared using supercritical carbon dioxide have also been reported [32].

Conventional drug DPIs are formulated either as loose agglomerates of micronized drug particles with aerodynamic particle sizes of less than 5 µm or as carrier-based interactive mixtures of micronized drug particles adhered onto the surface of large lactose carriers [33]. For local respiratory drug delivery, a particle size of 2 – 5 µm yields optimal benefit, whereas for systemic effects a particle size of less than 2 µm is a must for drug deposition in the deep lung peripheral airways. Particles greater than 5 µm may also result in systemic effects due to oropharyngeal delivery in the throat and subsequent oral absorption [34-37]. The powder formulation is aerosolized through a DPI device, where the drug particles are separated from the carrier (from drug-carrier mixtures) or the drug particles gets deagglomerated, and the dose is delivered into the patient's deep lungs. In these systems, particle size and flow property, formulation, drug carrier adhesion, respiratory flow rate and design of DPI devices extensively influence the performance [38]. Preparation of liposome by various techniques encapsulating the therapeutic agents is in a critical stage of development. Aggregation of the liposomes with other liposomes and carrier particles, thus producing powders with improper flow and dispersion characteristics, leading to poor lung deposition, has been a common problem during LDPF production. Various novel LDPF have been developed using freeze drying, spray drying, spray freeze drying or supercritical fluid technologies (**Figure 1**) to meet the need of enhanced powder performance, to overcome the known constraints of DPIs production and to achieve desired flow properties for efficient delivery of a wide variety of therapeutic agents. Improving drug

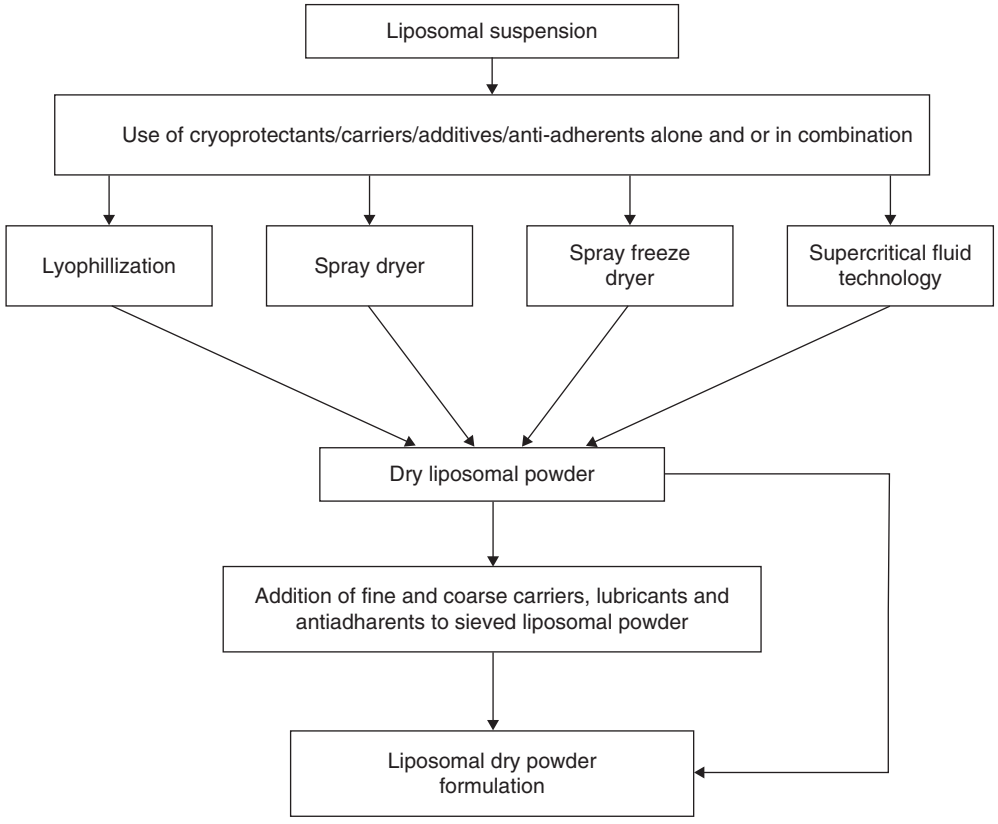

Figure 1. Formulation of liposomal dry powder formulation.

delivery to the lungs from a DPI formulation is possible by various techniques such as smoothing the carrier surface [39], reducing the particle size of the carrier [40,41] and using a ternary powder mix formulation [42]. The basic principal involves the preparation of a dry powder of liposomal drug by removal of water from the liposomal suspension after incorporation of small quantities of carriers (lactose, sucrose, etc), cryoprotectants (mannitol, trehalose, etc) and anti-adherents. Liposomal drug dry powder is then sieved successively from sieves # 100, 200, 350, 500. LDPF of this dry powder is prepared by mixing the dry liposomal powder with carrier lactose containing a blend of coarse and fine lactose in a specific sequence [43-45] and anti-adherents and lubricants for improving flow behavior of the powder, thus enhancing the lung deposition (**Figure 2**).

Freeze-drying and spray drying are the two major methods utilized to prepare LDPF. Freeze drying has been the method of choice for preparing LDPF, especially for liposomes encapsulating biologicals (proteins, peptides, enzymes, etc) for their higher stability [46]. Liposomal drug suspension with cryoprotectants and anti-adherents are freeze dried in different proportions to prepare LDPF with altered surface properties and density [43,46,47]. The therapeutic molecules, such as budesonide, ketotifen, amphotericin B, leuprolide acetate and levonorgestral have been formulated as freeze-dried LDPF for pulmonary administration [43,47-50]. Spray drying has been reported as a most favorable method for preparing micron-sized powders for pulmonary administration and has better control over particle formation and hence can be easily translated to large-scale production. Previously, DPIs were prepared by a spray drying process either by single and/or multiple emulsion technique or cosolvent systems, primarily consisting of an aqueous/organic solvent or mixture of aqueous and organic solvents [51]. Thermal degradation of the active ingredient is not a major concern as actual drying is at ambient temperature [52]. Spray drying of ethanolic solutions containing drug carriers and anti-asthmatic drugs has been reported [53]. Superoxide dismutase was encapsulated in the spray-dried liposomes to evaluate respirable properties [54]. Spray freeze drying of liposomal powder formulation containing ciprofloxacin showed good inhalation property [55]. In our laboratory, we have developed spray dried liposomal powder formulations of tacrolimus [56], dapsone [57] and amiloride hydrochloride [58] with good inhalation properties. Processing parameters during spray drying allows critical control over critical particle design features, such as particle size and distribution, surface energy, surface rugosity, particle density, surface area, porosity and microviscosity [30,57,58]. Control of these features has enabled new classes of therapeutics to be delivered by inhalation for better control of diseases.

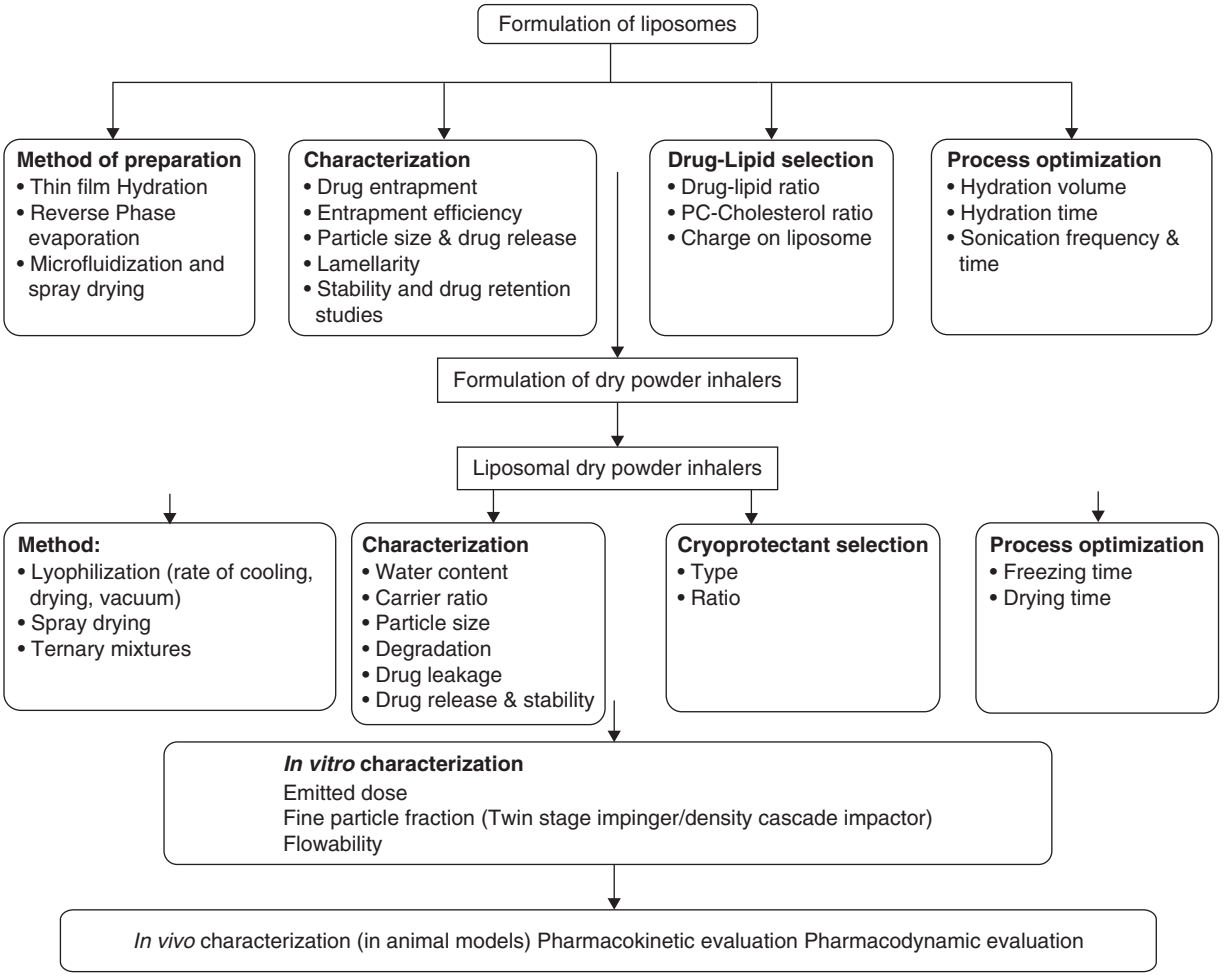

Figure 2. Process flow chart of LDPF formulation development.

Spray freeze drying involves spraying the drug solution into a freezing medium (usually liquid nitrogen) followed by lyophilization. Compared to spray drying, this process produces light and porous particles with enhanced aerosol performance, and the production yield is almost 100%. The method has been applied to prepare spray freeze-dried liposomal ciprofloxacin powder for inhaled aerosol drug delivery. In this study, the spray freeze-dried process that addresses many of the problems encountered by previous formulations and manufacturing methods has been used that relies on the spontaneous formation of liposomes. However, this is an expensive process and would only be justifiable for expensive drugs as it requires the additional use of liquid nitrogen and the freeze-drying step is more time consuming [55].

Recently, supercritical fluid technology (SCF) has been utilized in various pharmaceutical industrial operations including crystallization, particle size reduction, drug delivery preparation, coating and product sterilization [59,60]. It has also been a promising option in the formulation of particulate drug delivery systems such as nanoparticles and liposomes, which control drug delivery and/or enhance the drug stability and thus can be potentially used for formulation of DPIs [59,60]. The advantages of SCF technology include use of mild conditions for pharmaceutical processing (which is advantageous for labile proteins and peptides) and the production of particles with controllable morphology and narrow size distribution. Additionally, the complete removal of organic solvents can be assured by SCF technology [59-61].

In our lab, we have developed amphotericin B LDPF using SCF technology. Lipids and amphotericin B were dissolved in acidified methanol and chloroform known to have partial or full miscibility with an SCF antisolvent (typically CO_2). The liquid solution was then sprayed through a capillary or a nozzle into flowing SCF. The liquid solvent then dissolves into the antisolvent, while the lipids and drug do not. As a result, micron-size dry liposomes were formed. These liposomes may be further mixed with carrier lactose to deliver a suitable dose of amphotericin–B into the lungs [62].

The process flow chart for the formulation of liposomes, LDPFs, using different techniques and their evaluation characteristics has been shown below in **Figure 2**.

3. Inhaler device

DPIs overcome many of the problems inherent in pMDI design but suffer from limitations of their own, most notably a dependency on patients' inspiratory flow to generate a Fine Particle Fraction (FPF) suitable for deposition in the lungs. When choosing an inhaler device for an individual patient, it is important to check if patients are capable of generating sufficient inspiratory flow. The properties of devices help the patient to perform the correct inhalation and assure complete drug delivery to the lungs. Advanced devices may help to improve patient's compliance and thus improve drug delivery to lungs. Currently available DPI devices suffer from some inherent limitations associated with single dose devices, such as patients' incompliance does not allow for direct dose counting, the inhalation process sometimes has to be repeated until the capsule is empty, which may give rise to under-dosing and to high dose variability and associated with multiple dose device, need frequent cleaning, a device that uses a strip of foil drug containing blisters that cannot be reloaded, common patient errors, slow inhalation at the start of the inhalation maneuver, failure or difficulties to load the device before inhalation and exhaling into the device [63].

Various devices such as Rotahaler (Cipla Ltd, India), Inhalator brev ISF (Panacea Biotec Ltd, India) and spinhaler (Rhone-Poulenc Rorer Pharmaceuticals, USA) have been used by researchers delivering LDPF successfully to lungs [47-50,56-58]. However, the selection of inhaler device with appropriate features is paramount for successful delivery of LDPF.

The properties of an ideal inhaler device for delivery of LDPF are:

1. The inhaler device should possess control mechanisms which will ensure optimal respiratory flow at the time at which the dosage is triggered, a correct inhalation maneuver and allow the patient to verify successful completion of the inhalation maneuver.
2. The released active ingredient dosage and the deposition of the active ingredient in the lungs must be sufficiently high and reproducible.
3. It should be compatible with LDPF.
4. It should not alter the physical and chemical stability of LDPF.
5. Simple handling is a mandatory requirement.
6. It should have a dosage counter that counts not only the dosages but also the correctly executed inhalations and this characteristic would allow supervision of compliance.
7. It should be refillable.
8. It should have minimum maintenance requirements.
9. It should be patient compliant, and patient preference should also be taken into account.
10. The inhaler device should have a low-to-medium airflow resistance and require minimal patient coordination.

4. Liposome and LDPF – physicochemical characterization

The character of particulate systems is central to the performance of DPIs. The LDPF for pulmonary delivery may be evaluated at two levels as i) liposome; and ii) LPDF.

4.1 Characterization of liposomes

4.1.1 Percent drug entrapment (PDE)

PDE of the encapsulated drug in liposomes is vital for both high drug loading and cost management and is measured by separating the unentrapped drug from hydrated liposomal suspension. For water soluble drugs, the free drug is separated either by centrifugation at high speed to settle the liposomal pellet or passing through the Sephadex column. For water insoluble drugs, centrifugation at a low speed for settling the micron-sized free drug is carried out. However, use of Sephadex column ensures complete separation compared to low speed centrifugation. Then the liposomes were lysed with triton X 100 (0.1% v/v) and the drug was analyzed with a suitable analytical method [64,65].

4.1.2 Size and size distribution

The size and size distribution of liposomes before and after incorporation into LDPF were determined by particle size analyzers based on laser light scattering and the photon correlation principle [66,67]. The nanosized liposomes (< 200 nm) have shown better results in avoiding macrophage uptake and more uniform particle dispersion in LDPF [56]. Once incorporated into LDPF, the mean aerodynamic diameter of drug/drug containing carrier particles should not exceed 5 µm for pulmonary deposition.

4.1.3 Zeta potential

The zeta potential is an indication of the stability of the colloidal systems and indicates the charge present on the colloidal systems. High positive or negative surface charge on liposomes indicates higher stability because of the anticipated surface repulsion between similarly charged particles, hence inhibiting aggregation of the colloidal liposomal particles [56].

4.1.4 Morphology

The shape and lamellarity of liposomes has been evaluated using a polarising microscope and ^{31}P-NMR spectroscopy technique. Scanning electron microscopy and transmission electron microscopy are also widely used to study the size and shape of liposomes [68,69].

4.1.5 Entrapped volume

The entrapped volume of the liposomes, that is the aqueous entrapped volume per unit quantity of lipid (µl/µmol or µl/mg),

is of great significance for high entrapment of hydrophilic drugs. It is determined by entrapping a water soluble marker such as 6-carboxyfluorescein, ^{14}C or ^{3}H-glucose or sucrose and then lysing the liposomes by the use of a detergent such as triton X-100. Determination of the amount of marker that was trapped enables one to back calculate the volume of entrapped water. The entrapped volume is dependant on the lamellarity of liposomes. Higher lamellarity indicates high entrapment value. The entrapped volume can be enhanced using negatively and positively charged lipids so that the interlamellar distance may be enhanced for more water accumulation [43].

4.1.6 *Oxidative index*

The liposomes are characterized for per cent lipid oxidation at various time intervals indicating their stability. The oxidation of the lipids occurs by free radical chain mechanism in the presence of traces of transition metal impurity and specific oxidizing agents. The unsaturated lipids are more prone to oxidation than saturated phospholipids and their oxidative index is a measure of their oxidized state and hence indicating their stability [70-72].

4.2 Characterization of LDPF

4.2.1 *Flow behavior*

Angle of Repose is a very good tool for the determination of flow properties of a powder. If the angle of repose is below 25° then the powder will show excellent flow property; if it is in range of 30 – 35° then the powder shows good flow properties, and if it is above 40° then the powder will show poor flow properties. The Angle of Spatula is another tool that provides an indication of the internal friction between particles. The new Angle of Repose, which the material forms relative to the blade surface, is known as the Angle of Spatula. Generally, bulk solids with an Angle of Spatula less than approximately 40° are considered free flowing. The compressibility index is an indication of the per cent voids in the powder along with flow and dispersibility properties of formulation. Bulk and tapped density of the powder is determined for indicating the compressibility index and calculating the theoretical mean mass aerodynamic diameter [MMADt] of the powder with respect to its geometric diameter. The lower the density, the lower the MMAD of the powder. The MMADt was determined by the following formula:

$$\text{daer (MMADt)} = \sqrt{\rho} \times \text{d (VMD)} \quad (1)$$

where, ρ is tapped density in units of g/cm^3 and d is volume mean diameter in µm.

Dispersibility index is a measure of the propensity for agglomerates to disperse into fine particles on applying air pressure. It indicates the flow properties of the material. The more dispersible a material, the higher the potential for deaggregation [73].

4.2.2 *Moisture content determination*

Moisture content of the formulation may attribute stability, flow properties and thereby influence inhalation characteristics. Moisture content of LDPF is usually determined by the Karl Fischer Titration method [68,74].

4.2.3 *Reconstitution time and volume*

The small quantities of LDPF are reconstituted by vortexing in phosphate buffer (pH 7.4) and the time and volume necessary for reconstitution are noted [56].

4.2.4 *Drug retention and stability studies*

Comparative drug retention studies are performed on the LDPF for long-term stability at 5°C ± 3°C for 12 months and accelerated testing at 25°C ± 2°C/60% RH ± 5% RH for 6 months in accordance with ICH guidelines [75,76]. The product in its final packing is stored separately in all storage conditions. The samples of each batch stored at various storage conditions are withdrawn at definite time intervals, rehydrated with water for 30 min, and analyzed for size, zeta potential and per cent drug retained within the liposomes. The samples are also examined for evidence of caking and discoloration and per cent moisture uptake [75].

4.2.5 *Scanning electron microscopy photomicrographs and image analysis*

Scanning electron microscopy of the representative LDPF formulations is performed for determining the surface morphology, size and shape of formulation and to observe the aggregation property of liposomes with carrier particles [68,69]. Particle traits and surface topography are assessed using image analysis software. The traits such as roundness and aspect ratio can be determined as described below.

Roundness: reports the roundness of each object, as determined by the following formula: $(perimeter^2)/(4 * \Pi * area)$.

Aspect ratio: reports the ratio between the major axis and the minor axis of the ellipse equivalent to the object (i.e., an ellipse with the same area, first and second degree moments), as determined by Major Axis/Minor Axis [56].

4.2.6 *In vitro lung deposition studies*

The deposition of inhaled aerosolized particles to the targeted regions within the human respiratory tract can be estimated by employing the Anderson-Cascade Impactor (Official in USP) and Multiple Stage Liquid Impinger (Official in BP and EP). The method involves aerosolizing the LDPF placed in capsule using a suitable delivery device at vacuum of 28.3 liters per minute (lpm) (for 10 sec, in Anderson Cascade Impactor) and 60 lpm (for 5 sec, in TSI). The drug deposited at various stages of the equipment – indicating *in vitro* drug deposition at various lung regions – may be estimated by collecting the powder at each stage and subjecting it to drug content analysis using a suitable analytical method. From the drug deposition data

the emitted dose, fine particle dose, fine particle fraction (FPF), mass median aerodynamic diameter (MMAD) and geometric standard deviation (GSD) can be calculated [77]. Fine particle dose was calculated from the ratio of the total mass of powder (R) having a particle size below 5 µm found on the stages of apparatus and filter to the number of doses (n) aerosolized.

$$\text{Fine Particle Dose} = R/n \quad (2)$$

Fine Particle Fraction was calculated from the ratio of R to the total mass (ΣA) of powder delivered from mouthpiece of the inhaler into the apparatus:

$$FPF = R/\Sigma A \quad (3)$$

The MMAD of the particles was defined from log probability plot as the particle size at which the line crosses the 50% mark and the GSD was calculated from log probability plot as below:

$$\text{Geometric Standard Deviation} = (\text{Size X}/\text{Size Y})^{1/2} \quad (4)$$

Where size X is the particle size for which the line crosses the 84.13% mark and size Y is the 15.87% mark.

The other parameters estimated during analysis were: i) recoverable dose is the total quantity of drug recovered per capsule after each actuation; ii) emitted dose is the drug emitted from the inhaler device; iii) per cent emission is the ratio of emitted dose to total dose; and iv) per cent dispersibility is the ratio of per cent of FPD to emitted dose. The aerodynamic diameter below 5 µm indicates a formulation with high lung deposition. It is dependant on the formulation density and geometric size of the formulation.

Recent developments in the devices for determining the lung deposition and spray pattern evolved in Andersen 8-stage cascade impactor, next generation pharmaceutical impactor and model 3321 aerodynamic particle sizer aerosol spectrometer for evaluation of metered-dose or dry-powder inhalers and QA/QC testing of commercial inhaler products [78,79].

These devices are currently used to evaluate the pMDIs and DPIs and may prove to be a better tool for more accurate evaluation of the formulations for their *in vitro* lung deposition and the spray pattern.

4.2.7 *In vivo studies for determining lung absorption and disposition*

Irrespective of the large research interest in DPI drug delivery for local and systemic action, precise estimation of their pulmonary biopharmaceutics has proven to be a challenge. Currently *in vivo*, *in vitro* and *ex vivo* models are available for studying lung absorption and disposition of inhaled therapeutic molecules [80].

4.2.7.1 *In vivo approaches*

In vivo approaches in small rodents, mainly rats and guinea-pigs, are the most important assessment techniques due to acquisition of direct pharmacokinetic data, with relevance to reproducible dosing and control of lung regional distribution through the use of more sophisticated lung-dosing methods, such as forced intratracheal instillation, micro spray, nebulization and aerosol puff. Assessment of drug disposition after delivery to the lung is performed by estimating the drug in bronchoalveolar lavage and lung tissue homogenate. Intratracheal instillation, first described by Enna and Schanker, has been the most widely used technique for lung dosing because of its simplicity and accuracy [81]. Following general anesthesia, tracheotomy of the rats is carried out and aqueous solution or suspension containing test molecules is instilled into the airways through the tube using a microsyringe [82]. In the second method, instead of undertaking a tracheotomy, some studies have employed direct injection or spray of the dosing solutions into the lung through orotracheal intubation [83]. The recent development of custom-made dosing devices has now enabled liquid as well as powder administration into the lung in a relatively accurate and reproducible fashion [84]. The third method recognizes the importance of deep lung delivery to maximize systemic absorption by forced or ventilated administration with the animals on a surgical board at an inclination angle of 20 – 90° from the horizontal position [80]. In the fourth method, although neither anesthesia nor surgery is required, nose-only aerosol exposure, a common method in inhalation toxicology, has been rarely used in pulmonary biopharmaceutics research due to the fact that rodents are normally nose-breathing animals, thereby causing high nasal deposition in the nasal cavity and thus becoming a subject of mucociliary clearance to the gastro-intestinal tract (GIT), thus indicating parallel drug absorption from the nose and GIT as well as the lung, which is likely to lead to the overestimation of the lung's absorption capacity [82,85].

Irrespective of the technique used in the delivery of the drug to the lung, the estimation of drug levels after destructive tissue sampling is required for each data point, which requires a substantial number of animal sacrifices following intratracheal instillation. Collection of bronchoalveolar fluid (BALF) to determine the extent of drug released from liposomes in the lung for local action has been carried out [86]. The amount of drug estimated in liposomes from BALF indicates the amount of the drug still retained in liposomes and thus indicates the amount of drug metabolized or absorbed systemically [86]. Withdrawing multiple blood samples from an individual animal via surgical catheterization of an appropriate vein for serum collection allows collection of more important data. The drug level may be estimated using suitable analytical methods such as HPLC or bioassays to determine pharmacokinetic and pharmacodynamic response variables [47,56]. In our

laboratory we have developed validated analytical methods to estimate the pharmacokinetic profiles of Amikacin (Nicholas Piramal India Ltd, India), ketotifen, tacrolimus, amiloride hydrochloride, dapsone, rifampicin, isoniazid and amphotericin-b by measuring the drug levels released from liposomal suspensions in BALF fluid and lung tissue after intratracheal instillation in rats [43,45,48,49,56-58,86]. We have also measured serum LH levels to estimate the pharmacodynamic response generated after intratracheal administration of leuprolide and levonorgesteral liposomes [47,50].

All of these *in vivo* methods indicate evaluation of the pharmacokinetic and pharmacodynamic profile of the drugs in animals. However, these methods require sacrifice of animals and hence other techniques, including *in vitro* cell lines and *ex vivo* techniques, may also be used.

4.2.7.2 *In vitro lung epithelial cell models*

The *in vitro* cultured cell models are highly accepted and used in biopharmaceutical sciences to predict the absorption of test molecules from measurements of their apparent permeability coefficients (Papp) obtained from the *in vitro* cell monolayer model, alongside their solubility data [87,88]. Compared to an *in vivo* approach, such *in vitro* methods offer simplicity, robustness and better control in experiments and their data acquisition, in addition to the benefits of reducing operational costs and minimizing or eliminating animal sacrifice. Attempts have been made to develop a model for the airway-to-blood barrier with lung epithelial cell cultures over the past two decades. Primary cell culture of lung epithelium (tracheobronchial, bronchial, alveolar) has been developed from most species including rats [89-92] and humans [93,94], and several human lung epithelial cell lines derived from cancers, such as A549, Calu-3, 16HBE14o- and BEAS-2B, have been recently tested for their validity and usefulness [95]. By virtue of their continuous nature in culture, these cell line systems may offer more reproducibility and ease of use for investigators. Both of these primary and continuous culture models form lung epithelial cell monolayers, which enable determination of the transepithelial transport kinetics of test molecules (i.e., Papp), possibly for the purpose of predicting *in vivo* lung absorption. The *in vitro* cell culture models have been especially useful for the determination of cell uptake activity of the liposomally encapsulated drugs. The various parameters estimated during analysis using *in vitro* cell lines are cell uptake of liposomes, cytotoxicity studies of the liposomes with respect to plain drug solution and flow cytometric studies for cell death kinetics [95].

4.2.7.3 *Ex vivo lung tissue models*

Ex vivo tissue models have been preferentially employed in pulmonary biopharmaceutics research, when neither an *in vivo* nor an *in vitro* model has sufficiently clarified the mechanisms of drug transport or lung disposition kinetics. One of the most useful methods is isolated perfused lung (IPL) wherein the lung is isolated from the body and housed in an artificial system under certain experimental conditions to enable the separation of confounding whole body complications, for example distribution, metabolism and elimination, from lung-specific assessments. Such an isolated organ experiment maintains the architecture and functionality of the tissue and therefore must be a closer representation of the *in vivo* state than reconstructed *in vitro* monolayer models from a single cell type. For the lung disposition studies, an IPL prepared from small rodents has been most often employed [96-98]. Refinements of technique and recent kinetic modeling approaches have enabled the systematic delineation of complex and multiple lung disposition mechanisms. This (IPL) *ex vivo* model has been shown to be kinetically predictive of *in vivo*, with respect to macromolecular disposition, despite limitations concerning short viable periods of 2 – 3 h and the likely absence of tracheobronchial circulation.

5. LDPI for local and systemic delivery

Liposomes are believed to alleviate some of the problems encountered with conventional aerosol delivery due to their ability to: i) serve as a solubilization matrix for poorly water soluble agents; ii) act as sustained release reservoir; and iii) facilitate intracellular delivery of drugs, specifically to alveolar macrophages. Consequently, liposomes may also provide a means to prevent local irritation of lung tissue and reduce pulmonary toxicity, prolong local therapeutic drug levels, and generate high intracellular drug concentrations. Cumulatively, this would result in reduced systemic spillover and an increase in apparent drug efficacy. LDPF are convenient means of delivering liposomes in lungs for sustained action. LDPF have been actively explored by researchers due to their high potential for enhanced lung deposition and recently many articles have been published on LDPF for the treatment of various lung diseases such as asthma, chronic obstructive pulmonary disease (COPD), pneumonia, local fungal infection, lung cancer, cystic fibrosis and associated lung infections, local pain and pulmonary tuberculosis, which can be efficiently targeted by LDPF [1].

DPI of liposomal formeterol was developed to treat asthma using freeze-drying of liposomes prepared by thin film hydration method. The formulation showed enhanced FPF (48%) indicating high lung deposition of DPI [99]. In our laboratory, DPI formulation of liposomally entrapped Ketotifen Fumarate (KF) [100] and Budesonide [48] were developed. The DPI formulation consisted of liposomes of Egg PC and cholesterol prepared by lipid film hydration technique and sonicated to have the desired liposomal size (< 5 µm). Liposomal dispersion was subjected to lyophilization using sucrose as cryoprotectant. The formulations showed respirable fraction of 21.59 + 1.53% for KF and 20.69 ± 1.50% for BUD, which was comparable with that of the control 26.49 ± 1.52%, suggesting that these

liposomal formulations can be successfully delivered throughout the broncho-pulmonary tree [48,100]. In recent approaches, peptides such as vasoactive intestinal peptide (VIP) and its analog were assessed for their role in asthma and COPD as neurotransmitter or neuromodulator of the inhibitory non-adrenergic and non-cholinergic airway nervous system influencing many aspects of pulmonary biology. The peptides have been formulated as LDPF and tested for their potential benefits in COPD and asthma [101,102].

An effective management in prevention and treatment of *Pneumocystis carinii* pneumonia (PCP) associated with immunocompromised patients using LDPF encapsulated dapsone have been found to prolong drug retention in the lung and thereby achieve site-specific delivery [57].

Immunocompromised patients with cancer and AIDS generally have a tendency to develop invasive lung infections. These infections were efficiently treated by liposomal amphotericin b DPI [43]. We have developed amphotericin B LDPF using freeze drying, spray drying and SCF techniques (unpublished data), which have been compared for their efficacy in treating lung fungal infection. Recently, *in vitro* studies of liposomal amphotericin b developed by supercritical carbon dioxide-mediated anti-solvent process were also conducted [61]. Recent approaches to enhance alveolar macrophage uptake of these liposomes by coating them with alveolar macrophage-specific ligands (*O*-palmitoyl mannan, OPM, and *O*-polmitoyl pullulan, OPP) have showed enhanced macrophage uptake and better treatment [65]. Pulmonary delivery of liposomally encapsulated tacrolimus DPI for prolonged drug retention in the lungs as rescue therapy to prevent refractory rejection of lungs after transplantation has been investigated in our laboratory. It provides a practical approach for direct delivery of tacrolimus encapsulated in nanoliposomes for controlled and prolonged retention at the site of action. The developed LDPF of tacrolimus may play a promising role as rescue therapy in reducing the risk of acute and chronic rejection associated with lung transplant patients [56].

Cystic fibrosis and associated long-term infections can be treated by continuous prolonged antibacterial drug exposure in the lungs. Liposomal amikacin sulfate DPI was also successfully developed and found to have sustained targeted drug release in the lungs, which play a vital role in the management of cystic fibrosis. The effect of the addition of fine lactose (sorbolac 400) to enhance the lung delivery of LDPF of amikacin sulfate has been reported [45]. Liposomally encapsulated tobramycin [103] and gentamicin [104] are other formulations of choice for treating *Pseudomonas aeruginosa* infection in the lungs associated with cystic fibrosis. The liposomally encapsulated drugs showed sustained and controlled release at the site of action and hence showed better control over infection. In another investigation, inhalation delivery of spray freeze dried liposomal ciprofloxacin powder showed promising results for the treatment of pulmonary infections. The spray freeze drying manufacturing method for a ciprofloxacin formulation, the results of a reconstitution study, and the dispersion properties of the powder using a passive inhaler, have been discussed in this study. The formulation showed MMAD of 2.8 μm and FPF of 60.6%, indicating enhanced lung deposition for control of bacterial infection in cystic fibrosis [55]. In another approach, amiloride hydrochloride, a sodium channel blocker, a nano-LDPF has been developed which provides prolonged effective concentration in the airways to enhance mucociliary clearance by blocking sodium channels and preventing secondary infection in cystic fibrosis [58].

Liposome-encapsulated opioid analgesic agents delivered by the pulmonary route demonstrated superior local or systemic analgesia compared to that produced by the solution form of these agents administered by parenteral (i.v., intramuscular, or subcutaneous injection) or oral routes. It can provide rapid onset of analgesic action suitable for acute pain management, and will be inexpensive to manufacture. Along with acute rapid onset, sustained analgesia from continued release of liposome-encapsulated opioid (approximately 80 – 90% of the opioid dose) is an added advantage with the developed formulation. Thus, inhaled liposome-encapsulated fentanyl has been demonstrated to provide a significant advance in therapeutic activity against acute and chronic pain, at a lower cost than currently available therapies [105].

Liposomal antitubercular therapy for the treatment of pulmonary tuberculosis was developed for enhancing the therapeutic index of antitubercular drugs. Rifampicin encapsulated liposome for treating lung tuberculosis was prepared by the chloroform film method using different lipid and cholesterol ratios followed by freeze drying technique to obtain a dry powder for aerosol delivery. This approach helped the particles to reach alveoli without stimulation of immunological response and safely to alveolar macrophage. The LDPF showed better chemical stability than that of liposomal suspension form stored at 4°C and room temperature [106,107]. In another investigation, liposomal encapsulation of combination of tuberculostatic drugs, isoniazid, pyrazinamide, rifampicin, ethionamide and streptomycin in extruded liposomes designed for administration through inhalation was developed and evaluated for its performance [108].

The treatment of lung carcinoma by LDPF of anticancer agents has been a recent advance in pulmonary drug delivery. The LDPF of doxorubicin with cancer cell specific over-expressing transferrin ligand conjugated to the liposomes for enhanced cell uptake and reduced systemic toxicity by virtue of the reduced dose was achieved. The transferrin-conjugated doxorubicin-containing liposomes were tested on different cell lines as 16HBE14o, A 549, Calu-3 and were found to have higher cell uptake and higher cytotoxicity [95].

The majority of studies describing pulmonary delivery of proteins and peptides have focused on systemic delivery of

these drugs by targeting terminal bronchioles. The systemic delivery of liposomal suspension formulations of insulin and IL-2 had been found by researchers to have a promising outcome [109-111]. However, stability issues with these liposomal suspensions are likely to come. Although the use of LDPF has been well established for delivering small molecules, the local delivery of proteins is still little investigated. Liposomes of β-Glucuronidase (GUS), which has been used as a model protein to evaluate performance of inhaled dry powder liposomes demonstrated the feasibility of delivering these protein molecules to terminal bronchioles in therapeutic doses and offered the exciting possibility of aerosol delivery as dry powder formulations [46]. In another investigation, the effects of disaccharides and liposome carriers on the activity, solid-state characteristics, structural preservation and aerosol powder performance of spray dried superoxide dismutase (SOD) formulations was studied. Sucrose, trehalose and lactose were used as stabilizing adjuvants in the spray-drying process. Dipalmitoyl phosphatidylcholine (DPPC) was used as a major lipid component for preparing liposomes. The SOD\DPPC\sucrose formulation prepared by the spray drying process showed MMAD of 2 μm and SOD was found to be present in active form [112].

Although LDPFs have been established to deliver few local proteins and small molecules, they remain to be fully explored for systemic delivery. LDPF of leuprolide have been developed for systemic delivery and showed 50% bioavailability compared with the s.c. route. The studies justify the role of the pulmonary route as a promising alternative to the presently available SC route. The components of liposomal vesicles may be suitably changed to achieve higher bioavailability. Pulmonary delivery of leuprolide is expected to help in improving patient compliance, self-administration and avoiding the complications relating to injection procedure. The developed leuprolide DPI may be employed for both male and female contraception and treatment of prostate cancer in men and early puberty in children. In women it may be used for ovarian, endometrial, pancreas and breast cancer; endometriosis; uterine leiomyoma and anemia due to uterine fibroid tumors [47]. Similar contraceptive action was achieved after developing levonorgestral LDPF. The liposomes were developed by reverse phase evaporation technique and were freeze dried to develop DPI. The liposomal DPI was found to be effective in female rats for the contraceptive action [50].

A review of recent patents claiming the use of LDPF prepared by different approaches to improve lung deposition will help to decide the focus of the research in the area of technological gaps. Various patents have also been filed in the area of LDPF throughout the world, and the most relevant with respect for formulation technology have been listed in **Table 1**.

The recent patents were filed for preparation of LDPF based on aerodynamically light, porous, monodisperse formulation prepared using freeze dried liposome cakes followed by jet milling, spray drying, spray freeze drying and supercritical fluid processing, for treatment of both local as well as systemic drug delivery (**Table 1**). The inherent advantages, recent patents and ongoing research in area of LDPI suggest that delivery of liposomes in dry power form has a great promise for delivering therapeutic agents (drugs/protein/peptides) to the lungs for local and systemic action.

6. Clinical trials

The first clinical investigation of systemic insulin delivery via the lung took place in the 1920s, and the interest in this route has increased in recent years with the advances made in formulation and recombinant technology. The protein for inhalation currently available on the market is DNase, but a growing number of proteins/peptides are under various phases of clinical trials. DNase is a phosphodiesterase capable of hydrolyzing polydeoxyribonucleic acid and provides effective delivery of enzymes such as lytic agents like DNase to sites of pulmonary distress in the management of pneumonia, bronchitis, cystic fibrosis, or emphysema. Liposomal encapsulation provides better stability to DNase in DPI form [123]. Systemic inhaled insulin formulation was recently launched on the market and subsequently withdrawn due to marketing reasons, but it has been a big impetus to the pulmonary delivery of macromolecules. The other proteins and peptides in Phase III trials include leuprolide and gamma-interferon [115]. Various clinical trials for evaluating the efficacy of liposomal encapsulated drugs are discussed below. Typically, liposomal formulations have been delivered by nebulization, where suspension formulations are atomized in reported *in vitro*, preclinical and even under clinical studies [124]. We summarize the ongoing and completed clinical trials conducted with liposomal suspension below. The success of these liposomal formulations will have an important impetus on the development of LDPF for both local as well as systemic delivery of various therapeutic drugs, proteins and peptides.

Of the liposomal suspensions for the treatment of disorders associated with cystic fibrosis, Amikacin has been granted orphan drug status by the US Food and Drug Administration (FDA) and The European Medicines Agency (EMEA) for the treatment of pulmonary infection due to *P. aeruginosa* and additional Phase II and III studies are also being performed [125]. Recently, Transave announced positive Phase II results for once-daily Arikace™ (Transave Inhalation Biotherapeutics, USA; liposomal amikacin for inhalation) in the treatment of cystic fibrosis (CF) patients who have pseudomonas lung infections. Arikace can be delivered through nebulization, which enables the small aerosol droplet size (~ 3.0 μm) to facilitate more effective distribution in the lungs. In addition to clinical studies in CF patients with Pseudomonas lung infections, clinical development has been initiated in non-CF

Table 1. Patents filed in the area of LDPFs for pulmonary delivery.

Serial no.	Title of patent	Description	Ref.
1	Liposome powders	This invention, for the first time, provides liposomes in a free-flowing dry powder form for delivering the encapsulated drugs and other compounds in formulations such as pills, crèmes, gels and powders which have not previously been possible. Specifically, the invention describes that freeze dried liposome cakes after jet milling produces particles in the size range of 1 – 100 µm, which produces a free-flowing dry powder. The flow properties of these liposome powders can further be improved by mixing the liposome powder with carrier powders such as spray dried lactose, other carbohydrates and carbohydrate alcohols such as mannitol or sorbitol, with cellulose or with silica derivatives with a size range of, for example, 40 – 100 µm diameter for pulmonary delivery	[113]
2	Engineered monodisperse inhalation powders for effective treatment of lung diseases	The invention provides a monodisperse powder aerosol formulation comprising the therapeutic agent and biocompatible carriers with superior aerosol properties for deep lung and/or airways delivery	[114]
3	Aerodynamically light porous dry powder inhaler (ALPDPI) formulations for targeted pulmonary deposition	ALPDPI formulations having low tap density below 0.4 g/cm^3 and a geometric diameter between 5 and 30 µm and having targeted and enhanced pulmonary deposition with prolonged residence time, and the method of preparation and administration thereof are provided for the prophylaxis/treatment/diagnosis of various pulmonary and systemic disorders. The ALPDPI formulations comprising of vesicles/particles offers the advantage of altering favorably the pharmacokinetic profile of the bioactive agents, which helps in effective management of pulmonary and systemic disorders. The ALPDPI formulations of bioactive agents may be effectively aerosolized alone or co-administered with a coarse carrier for administration having enhanced FPF/respirable fraction to the specific sites of lungs in the effective prophylaxis/treatment/diagnosis of pulmonary or systemic disorders by using a high/medium/low resistance device	[115]
4	Preparation of amphotericin b liposomes by supercritical fluid technology	The patent discloses the development of dry liposomes of amphotericin-b by a supercritical antisolvent technique for treatment of invasive lung fungal infection	[62]
5	An inhalation system	The patent discloses a liposomal inhalation system of DPIs for aminoglycoside such as gentamycin, amikacin, tobramycin, neomycin, kanamycin and netilmicin for the treatment of lung infections	[116]
6	Methods for treating lung cancer	The patent discloses the methods and a DPI formulation of liposomal cisplatin, carboplatin and a taxane by inhalation of a lipid composition for treatment of bronchoalveolar carcinoma, carcinomatosis with lymphangitic spread or primary and metastatic lung carcinoma	[117]
7	Powders for inhalation	The invention described proliposomal powder compositions of a biologically active compound in particulate dispersion in a lipid for inhalation. A process of manufacture of proliposomal powder comprising single phase discrete particles of a biologically active component, together with a lipid or mixture of lipids having a phase transition temperature of below 37°C for inhalation	[118]
8	Lipid formulations for spontaneous drug encapsulation	A patent disclosing a DPI formulation comprising a lipid and an active agent having a liquid phase transition temperature of less than or equal to 37°C on hydration and a liquid phase transition temperature of greater than 57°C in dry form. On inhalation the drug spontaneously encapsulates into lipid inside the lungs. This formulation was investigated in treatment of anthrax infection on inhalation	[119]
9	A dry powder formulation for gene therapy	The invention relates to methods and compositions suitable for gene therapy, particularly in compositions of DNA, RNA, oligonucleotides and proteins complexed with lipid and delivered in a concentrated and convenient form, either as a freeze dried powder delivered as a DPI formulation or in liquid form after reconstitution in a suitable aqueous medium	[120]

Table 1. Patents filed in the area of LDPFs for pulmonary delivery (continued).

Serial no.	Title of patent	Description	Ref.
10	Liposomes containing a corticosteroid	A pharmaceutical composition comprising an active ingredient (corticosteroids) contained in liposomes or dehydrated liposomes	[121]
11	Spray freeze dried liposomal ciprofloxacin powder aerosol drug delivery	The invention describes spray freeze dried liposome particles encapsulating a biologically active agent, such as an antibiotic – ciprofloxacin, within a phospholipid, and a method of producing a powder for inhalatory aerosol delivery by mixing a biologically active agent with a phospholipid to form a liquid liposome suspension; and spray freeze drying the liposome suspension to form particles of powder	[122]

bronchiectasis patients with Pseudomonas lung infections [126]. Thereafter in July 2008, Aradigm reported successful Top-Line Phase II Data with Inhaled Liposomal Ciprofloxacin for Cystic Fibrosis and published positive results from an open-label, two-week efficacy and safety study of its once-daily inhaled liposomal ciprofloxacin in patients with CF. In this trial the liposomal ciprofloxacin was well tolerated, and no serious adverse effects were observed [127]. In June 2008, Aradigm Corporation (USA) announced initiation of a multicenter Phase II clinical trial of its inhaled liposomal ciprofloxacin in adult patients with non-CF bronchiectasis [128].

Of liposomal suspensions for the treatment of fungal disorders, Nebulized Liposomal amphotericin b (Ambisome® [Astellas Pharma Inc, USA]) for Prophylaxis of Invasive Pulmonary Aspergillosis (AMBINEB) has also been investigated. This study is currently recruiting participants for a Phase II trial (verified by PETHEMA Foundation) [129]. In another investigation, a clinical Phase III trial studying the steady-state concentrations of inhaled liposomal amphotericin b (Ambisome) in lung transplant recipients via aerosolized nebulization has been completed at the University of Pittsburgh Medical Centre, Pittsburgh, Pennsylvania, USA. In this trial the characteristic pharmacokinetic profile of liposomal amphotericin b was achieved in the serum and epithelial lining fluid of the lung with four doses of liposomal amphotericin b administered via aerosolized nebulization in lung transplant recipients [130]. A Phase II/III randomized double-blind study comparing the safety and the efficacy of a weekly administration of 25 mg nebulized Ambisome with a nebulized placebo solution to prevent invasive pulmonary aspergillosis in neutropenic hemato-oncologic patients has also been completed at Erasmus Medical Centre, Netherlands, sponsored by Gilead Sciences and Nexstar Pharmaceuticals [131].

Of liposomal suspensions for the treatment of lung cancer, the clinical trials on aerosolized liposomal camptothecin in patients with metastatic or recurrent cancer of the endometrium or the lung were completed in 2005 at University of New Mexico, USA. This clinical trial was conducted to evaluate the arterial concentration of 9-nitrocamptothecin (9NC) administered by inhalation in comparison to venous and urine concentrations, to determine the concentration of 9NC in the alveolar fluid over time and also to determine the tumor concentration of 9NC administered by inhalation [132]. Among clinical trials recruiting participants, the University of New Mexico, USA is conducting a Phase II clinical trial based on Aerosolization of Liposomal 9-Nitro-20 (*S*)-Camptothecin (L9NC). The purpose of this clinical trial is to determine the overall response rate to L9NC administered by aerosolization in patients with non-small-cell lung cancer. This trial also aimed to determine the toxicity profile of L9NC administered by aerosolization, and to perform a pharmacology study of L9NC in the plasma and the lungs after aerosolization [133]. Another Phase II clinical trial of aerosolized L9NC for the management of Corpus Uteri Endometrial Cancer sponsored by the University of New Mexico, USA has also been recruiting patients. The purposes of this study are to study the efficacy of the formulation in patients with metastatic endometrial cancer and to establish a toxicity profile and pharmacological profile after aerosolization [134]. The Phase I study was performed recently to test the feasibility and toxicity of administering interleukin-2 (IL-2) liposomes nebulized using a Puritan Twin Jet Nebulizer (Puritan-Bennett Corporation, USA) and a standard compressor to patients with pulmonary metastases. This clinical trial concluded that the delivery of IL-2 liposomes by inhalation is well tolerated [135].

Among liposomal suspensions in the management of pain, a Phase I clinical trial designed to assess single-dose and multiple-dose pharmacokinetics and safety parameters utilizing a dosage of 3 ml (500 µg/ml) AeroLEF delivered via nebulization with the AeroEclipse BAN device was completed YM BioSciences, University of Toronto, Canada. This trial has been conducted to test the subjects who were not blocked with naloxone or other opioid receptor antagonists [136]. Another pilot Phase I trial has been completed evaluating two-period bioavailability, safety and pharmacokinetic profile after a single dose of i.v. fentanyl (200 µg) and single doses of 2 ml or 3 ml inhaled AeroLEF (500 µg/ml) delivered by nebulization with the AeroEclipse BAN device administered in normal healthy non-smoking subjects by YM BioSciences, University of Toronto, Canada [137].

For evaluation of the efficacy of liposomal encapsulated therapeutic agents for the management of various disorders, various clinical trials have been completed and several more are ongoing. Liposomes delivered as LDPF have shown promising results in both *in vitro* and pre-clinical investigations. However, to date no LDPF has been studied under clinical studies. Concerns arising from drug leakage and stability during storage and aerosolization of a liposomally encapsulated agent using nebulizers can be overcome by the use of LDPF. Hence, the delivery of LDPF via the inhalation route holds great promise for treatment of both local and systemic disorders.

7. Inhalation toxicology

Liposomal dry powder formulations may possess highly useful and novel properties for pulmonary drug delivery, but safety considerations remain of paramount importance since completely inert materials have the ability to exhibit toxic effects by virtue of a reduction in their size and an associated increase in surface area : mass ratio [138]. In addition, the manipulations in the synthetic lipids used to prepare liposomes may impart the toxicity. A safety evaluation of the ultrafine liposomes requires the collaborative efforts of toxicologists (animal, cellular, molecular), clinicians (pulmonary) and environmental scientists [139]. These nanoliposomes may have potential effects on respiratory mucosa, causing lung and systemic inflammation to the cardiovascular system and central nervous system upon prolonged exposure [140]. Although approaches to evaluate general organ toxicity and inhalation toxicity are similar, there are specific challenges in the toxicity testing of inhaled pharmaceuticals. The toxicology of LDPF (particulate matter) differs from the toxicology of substances as the LDPF composing chemical is either soluble or not soluble in biological matrices, thus influencing greatly the potential exposure of various internal organs. This may change from a high local exposure in the lungs and a low or neglectable exposure for other organ systems after inhalation, although absorbed species may also influence the potential toxicity of the inhaled particles [141]. The potential interaction with tissues and cells, and the potential toxicity, greatly depends on the actual composition of the LDPF. Determination of the inhaled dose is more difficult than with i.v. or oral doses. The correlation of the animal dose with the human dose is also more difficult for inhalation exposure than for other administration routes. Additionally, inhalation of pharmaceuticals by the nose (by animals) and mouth (by humans) imparts significant differences in results. Thus there is a significant challenge in exposure methodology and technology, which requires novel approaches involving alteration to particle size of the agent or dosing procedure [140]. Because the respiratory tract is the site of deposition, local respiratory toxicity and possible damage to lung cells needs to be assessed. The histopathology of lung sections must be evaluated carefully, including sections of all major anatomical regions including alveolar, tracheobronchial and head airway regions. Nasal toxicity in animals is also of paramount importance, because in animals the cells in the nose receive the highest dose per unit surface area of any cell type in the respiratory tract. Therefore, this region can provide a sentinel function in assessing the potential cytotoxicity of inhaled compounds [140]. Bronchoalveolar lavage coupled with analysis of cytology, enzymes and mediators can be a useful method to assess respiratory tract toxicity [142]. Special studies on pulmonary function, mucociliary clearance, or immune response may be needed, depending on the nature of the inhaled pharmaceuticals [140]. Liposomes in nanosize can cause new types of effects not previously seen with larger particles (e.g., mitochondrial damage, uptake through olfactory epithelium, platelet aggregation and cardiovascular effects). These findings must be considered in developing toxicological studies. Systemic toxicity also needs to be evaluated and may be an issue in some cases, despite the fact that liposomal encapsulated drugs are for local action in the majority of cases [141].

8. Expert opinion

Many of the developments in LDPF for pulmonary delivery represent tremendous progress over the past decade. Liposomes provide an efficient delivery system for the treatment of pulmonary disorders due to their biocompatibility, biodegradability and relatively non-toxic nature [17]. Administration of liposomes to the respiratory tract is particularly attractive because of the accessibility of the lung as a target organ, the compatibility of liposomes and lung surfactant components (85% phospholipid), and the need for sustained local therapy following inhalation [20]. Liposomal drug encapsulation has been shown to be promising in releasing the drug in a controlled fashion if delivered selectively in various regions of the lung. Its pharmacological response is not only prolonged, but also improves the therapeutic index of the drug because of enhanced intracellular delivery and slow systemic dilution and clearance [18]. Unlike other nanocarriers, its residence time in the lung is sufficiently prolonged in spite of its composition being more biocompatible and non-immunogenic [19]. When the liposomal encapsulated drug is delivered into the deep lung, it reduces the rate of systemic dilution especially needed for protein, peptide-based drugs and not expected to cause any damage to lung tissue. As a drug delivery system, liposomes can significantly alter the pharmacokinetics and pharmacodynamics of entrapped drugs [18,19]. It is necessary to understand that liposomal size and size distribution, composition, site of delivery, the physicochemical nature of the drug, cell fusogenic agents, conjugation of ligand for selective receptor/transporter mediated delivery, etc, all play an important role in maximizing the therapeutic index, minimizing/eliminating side effects, reducing

dose/frequency of dosing and possibly drug resistance, systemic toxicities and the cost of therapy [21,22].

Although liposomes are instable in suspension form, long-term stability can be achieved by processing into powder form using a freeze dried or spray dried technique. The dry liposomes encapsulating therapeutics can be delivered to lungs as a DPI, by virtue of its propellant free nature, flexibility in formulation development and high patient compliance. However DPI are complex in nature and their performance relies on many aspects, including the design of the inhaler, the powder formulation and the airflow generated by the patient [24-26]. Liposomally encapsulated drugs, by virtue of their controlled and intracellular delivery, can be delivered to treat pulmonary disorders such as asthma, interstitial lung disease and COPD (corticosteroids), lung cancer (anticancer agents), local pulmonary infections and cystic fibrosis (aminoglycosides and other antibiotics) lung transplant rejection (immunosuppressants), invasive lung fungal infection in immunocompromised patients (amphotericin b), pneumonia (antibacterial agents), pulmonary tuberculosis (antitubercular agents) and systemic delivery of peptides and proteins. Delivery of drugs to the lungs includes cellular targeted delivery, which can improve efficacy and reduce unwanted systemic side effects. Various reports in the recent literature have suggested improved therapy through modification of the surface chemistry of liposomes. Coupling liposomes to a ligand – that is directed towards an overexpressed receptor in cancer cells and that normally undergoes endocytosis – is a strategy that can improve selectivity and facilitate access of liposomes to the intracellular compartment. Gene therapy studies have already started using cell-selective gene promoters to target genes to specific lung cell types. Targeted immunoliposomes in DPIs also give intracellular targeting. DNA delivery is also included, as much of the recent research into intracellular and cell type specific ligands has been based on the development of gene delivery vectors. LDPFs also offer the opportunity for systemic administration of peptides and proteins that are at present usually administered parenterally. It is expected that the further research interest in this route of administration will lead to more breakthroughs in areas of both formulation and device design, thereby benefiting patients and thus the market will improve. The preferred mode of administration of liposome formulations is dry powder due to the high lung deposition, drug loading capacity and stability. However, recently the inhalation of liposomal drugs after reconstitution of LDPFs using a nebulization technique has shown better compliance and ease in delivery to younger and older patients. However, the same compliance may be achieved with active DPI devices with the added advantage of higher pulmonary deposition, that is 20 – 25% in nebulization as compared with 50 – 75% through dry powder form.

In the last decade, many liposomal formulations have reached the stage of clinical trials for the treatment of pulmonary distress, cystic fibrosis and lung cancer. These formulations have given very promising results in both *in vitro* and *in vivo* studies. However, several key issues such as patient compliance and education, cost of treatment, stability and large scale production of liposome formulations, etc, need to be addressed in order for a route to be found from theory to clinical reality. Typically, liposomal formulations have been delivered by nebulization, where suspension formulations are atomized and tested *in vitro*, preclinical and even under clinical studies. LDPF has shown promising results in *in vitro* and preclinical investigations. However, to date LDPF has not been studied under clinical studies. Concerns arise when nebulizers are used to deliver a liposomally encapsulated agent, from a drug stability and leakage perspective. To circumvent these issues, LDPFs hold promise, and as the lungs are vital organs, a major research contribution into DPIs is still to come through the development of better formulations and devices especially suited to LDPFs to improve the lung deposition of liposomally encapsulated drugs. When choosing an inhaler device for the aerosolization of LDPF, it is important to check compatibility with product, stability of formulation during aerosolization, delivery efficiency, capability of patients to generate sufficient inspiratory flow and patient compliance. Ongoing research in the area of liposome and dry powder technologies and active device design should improve the clinical outcomes and continue to expand therapeutic options as newer inhaled drugs become available. Further developments focused on liposomal delivery in dry form to the lung are therefore warranted.

Although there are advances in area of liposomal DPIs, inhalation toxicity testing of the formulations is of prime importance. Inhalation toxicity studies require definition of delivered dose to the respiratory tract, in humans and animals, which is more difficult than with other routes of exposure. To use the potential of LDPF in drug delivery, full attention is required to safety and toxicological issues. Understanding of clearance kinetics of inhaled LDPF will also be important in understanding their potential for adverse effects. Modifications to new therapies for respiratory diseases and systemic delivery will provide new challenges to conducting well-designed inhalation toxicology studies to support these products.

Declaration of interest

The authors state no conflict of interest and have received no payment in the preparation of this manuscript.

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