EXPERT OPINION

Lipid-based carriers: manufacturing and applications for pulmonary route

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Introduction: Recent advances in aerosol therapy have sparked considerable interest in the development of novel drug delivery systems for pulmonary route. Development of colloidal carriers as pharmaceutical drug delivery systems has spurred an exponential growth; the encapsulation of bioactive molecules into relatively inert and non-toxic carriers for *in vivo* delivery constitutes a promising approach for improving their therapeutic index while reducing the side effects. Extraordinary success has been made toward improving efficacy by developing lipid-based carriers (LBCs); among classical examples are liposomes and solid lipid nanoparticles (SLNs).

Areas covered: The authors review lipid-based colloidal carriers – liposomes and SLNs – as pulmonary drug delivery systems. Conventional methods of liposome preparation and recently developed systems are discussed. Special attention is given to SLNs and their main manufacturing techniques. Finally, a summary of recent scientific publications and important results in the field of pulmonary lipidic carriers are presented. Some practical considerations regarding the toxicological concerns of such systems are briefly cited.

Expert opinion: Despite several scientific investigations, numerous advantages and encouraging results, LBCs for pulmonary route have attained only few great achievements as many challenges still remain. Problems limiting the use of such system seem to be the complexity of the respiratory tract as well as the lack of toxicity assessment risks of colloidal carriers.

Keywords: aerosol, liposomes, lung, pulmonary drug delivery, preparation methods, solid lipid nanoparticles, therapeutic applications

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1. Introduction

Growing attention has been given to the potential of pulmonary system as a non-invasive administration route of therapeutic agents in prophylaxis, treatment and diagnosis for both local and systemic disorders [1]. Due to the promising anatomical features of the lung, especially the large absorptive surface area (up to 100 m^2), the extremely thin mucosal membrane (0.1 – 0.2 μm) and the well-developed vascular system [2], pulmonary route offers many advantages for drug delivery. Further advantages in systemic drug delivery via lungs included the absence of the hepatic first-pass metabolism, the relatively low enzymatic activity for drug-metabolizing within intracellular or extracellular region [3], the very rapid onset of action [4] and the high drug bioavailability. To be effective, pharmaceutical aerosols have to be deposited in the targeted site at a therapeutic dose within the lungs. Once deposited, the fate of inhalable drugs in the pulmonary system depends on the dynamic interaction of the anatomy of the respiratory tract, the physiological clearance mechanisms operating within the lung and the physical characteristics of the formulation [5]. The respiratory-defense system, including mechanical (air filtration

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Article highlights.

- The lungs seem to be the most historic route of drug delivery; aerosol technology is a potential route for the delivery of therapeutic agents for the prophylaxis, treatment and diagnosis of both local pulmonary and systemic diseases.
- Lipids are a class of compounds including natural molecules; their use in drug-carrier systems such as liposomes or solid lipid nanoparticles (SLNs) is well established and has attracted considerable research effort for pulmonary drug delivery.
- Lipid-based carriers (LBCs) offer many advantages over traditional aerosol formulations; encapsulation has been suggested as a way to enhance the bioavailability, optimize the residence time and the release profile, reduce the pulmonary clearance and toxic side effects.
- Major limitations of the use of such system seem to be the complexity of the respiratory tract as well as the lack of toxicity assessment risks of colloidal carriers.

This box summarizes key points contained in the article.

mucociliary clearance), chemical (antioxidants, antiproteases and surfactant lipids) and immunological elimination mechanisms [6,7], must be considered as a physiological barrier that actively purge the deposited particles within lungs [5]. Accordingly, the development of appropriate drug delivery systems has become an important issue that must take into account all these factors. The fundamental requirement was that inhaled formulation must have well-defined properties specifically the particle size [8], the density, the mass median aerodynamic diameter (MMAD) [9,10] and the respirable fraction (RF); particle or droplet size seems to be the most important factor determining the site of aerosol deposition [11]. Otherwise, the drug delivery system should improve therapeutic properties such as increasing drug efficiency, optimizing residence time and release profiles and enhancing drug targeting to the diseased site within lung.

Colloidal carrier systems occupy unique position in new drug delivery technologies leading to most optimized therapeutic outcomes. It has been confirmed that the encapsulation of bioactive molecules into relatively inert and non-toxic carriers for *in vivo* delivery constitutes a promising approach for improving their therapeutic index while reducing the side effects, which have created an attractive and efficient approach for pulmonary drug delivery. Extraordinary success has been made toward improving efficacy by developing lipid-based carriers (LBCs); among classical examples are liposomes and solid lipid nanoparticles (SLNs). LBC has the potential to increase and sustain the release of the therapeutics locally or systemically. Furthermore, targeting the site of action could decrease the required body dose and reducing systemic side effects [1].

Liposomes, vesicles composed of phospholipids with or without cholesterol, are capable of entrapping an extensive range of molecules [12]. Liposomal aerosols are believed to alleviate some of the problems encountered with conventional aerosol delivery and offer the advantage of uniform deposition of active drugs, increased potency and reduced toxicity [13]. Typically, liposomal formulations have been delivered by nebulization. But several concerns raised from drug stability and leakage perspectives when nebulizers are used. To avoid these issues, dry powder formulations have been developed; lyophilization, jet milling and spray-freeze-dried (SFD) processes were used to manufacture liposomes-based powder formulations [14].

On the other hand, SLNs emerged as a promising non-toxic nanocarrier for drugs in 1990s, the superior physicochemical characteristics, such as small size, biocompatible composition and deep-lung deposition ability, make them more suitable as carrier for pulmonary drug delivery than their predecessors [13]. Dual effect of prolonged drug release and rapid drug transport could be achieved by means of SLNs [15].

This paper is giving a review about lipid-based colloidal carriers, liposomes and SLNs, as drug delivery system for pulmonary route. The first section is describing the most widely used methods for liposomes preparation, with a special focus on the new developed techniques. In the second section, a special attention will be dedicated to SLNs and the main manufacturing techniques. Finally, the last section will summarize the recent scientific research as well as the important result in the field of pulmonary LBCs. Some practical considerations regarding the toxicological concerns will be briefly cited.

2. Liposomes

2.1 Definition

Liposomes, a colloidal drug delivery system, are defined as nanosized spherical vesicles first introduced by Bangham and Horne [12]. They are composed of one or more phospholipid bilayers enclosing an aqueous compartment and are spontaneously obtained under phospholipids hydration in aqueous solution [16]. The vesicular structure provides to liposomes the ability to load either hydrophilic drugs in the aqueous core or lipophilic compounds within the lipid bilayer; amphiphilic substances were entrapped at the bilayer interface [17,18].

Liposomes are mainly composed of natural or synthetic lipids that are considered to be relatively biocompatible and biodegradable material. Other constituents such as cholesterol and hydrophilic polymer-conjugated lipids may be added to the membrane composition in order to alter stability or kinetics [19]. Extensive testing of phospholipids safety has been made for pulmonary route; the effects of inhaling drug-free liposomes [20], fluorescein-labeled liposomes [21] and beclomethasone dipropionate-liposome [22] in healthy volunteers reported no untoward side effects as well as excellent tolerance in volunteers. Results proved them to be remarkably safe for pharmaceutical use.

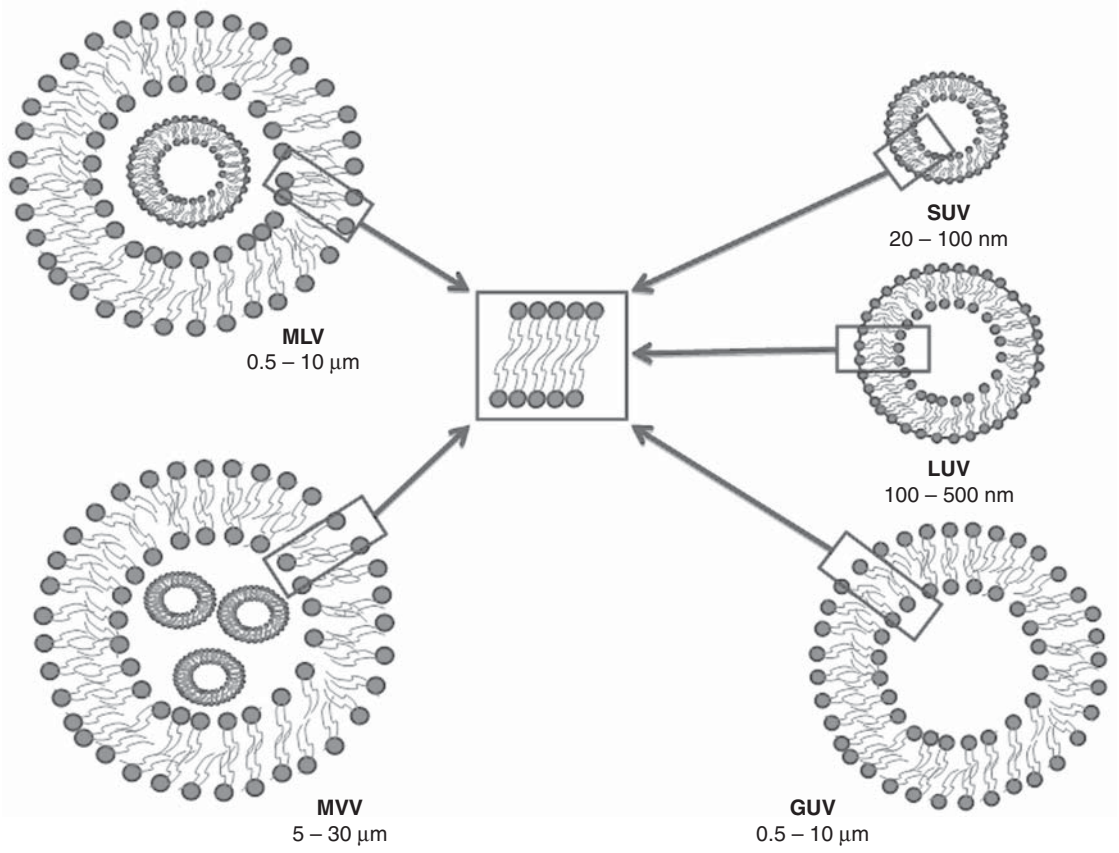

Figure 1. Liposome classification according to their lamellarity (uni-, oligo- and multilamellar vesicles), size (small, intermediate or large).

LUV: Large unilamellar vesicles; MLV: Multilamellar vesicles; MVL: Multivesicular liposomes; SUV: Small unilamellar vesicles.

2.2 Classification

Liposomes can be classified on the basis of their size and bilayers number, into (**Figure 1**): multilamellar vesicles (MLV) 1 – 5 μm; large unilamellar vesicles (LUV) around 1 μm; small unilamellar vesicles (SUV) and multivesicular liposomes (MVL). MVL are characterized by their structure of multiple non-concentric aqueous chambers surrounded by a network of lipoidal membranes [23]. They contain a neutral lipid (triolein, tricaprylene, trilaureine, tributyrine) as an integral component which allows the formation of this unique multivesicular structure [24].

2.3 Preparation process

Excellent reviews on the preparation and physicochemical characterization of liposomes including conventional [19,25] and novel techniques [26,27] are available.

2.3.1 Conventional techniques

There are a wide variety of conventional techniques that can be used to produce liposomal formulations. The original method of Bangham and Horne also called the thin-film hydration procedure appears to be the most simple and widely used method [12]. Accordingly, a lipid phase was prepared by dissolving an accurately weighed quantity of the lipids in the organic solvent using a round-bottom flask. The solvent mixture was removed from the lipid phase by rotary evaporation resulting on the thin film of lipids. The dry thin film was hydrated with an aqueous buffer at a temperature above the lipid transition temperature (Tc) [28]. Regrettably, this method produces a heterogeneous population of MLV (1 – 5 μm diameter) and has limited use because of its low encapsulation ability and the impossibility to produce in large scale.

Conventional methods also included the reverse-phase evaporation technique [25], the ether or ethanol injection technique [29-32], the detergent dialysis [33,34], the calcium-induced fusion and the freeze-thaw method [35]. These preparation techniques can be said to involve mainly three stages: lipids dissolution in organic solvents, the dispersion of the lipidic phase in aqueous media and the purification of the resulting liposomes via some complex processes, such as gel permeation chromatography and/or centrifugation. These techniques mainly yield LUV or MLV. Thus, further steps (e.g., high-pressure homogenization (HPH), ultrasonication, extrusion through polycarbonate filters, etc.) are needed to produce small and narrowly size distributed dispersions of SUV.

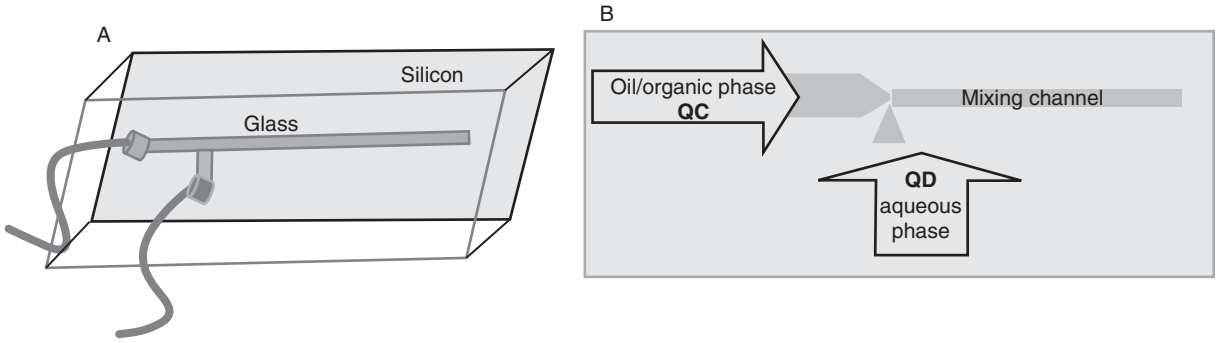

Figure 2. A. A schematic illustration of the microfluidic T-junction device. **B.** A top view of the microfluidic T-junction device in a two-dimensional representation.

Qc: The continuous fluid (oil or organic phase) flow inlet the mixing channel; Qd: The dispersed fluid (water or aqueous phase) flow via the orthogonal inlet; Qd and Qc: Inlet velocity.

The disadvantages that all the conventional liposome-manufacturing methods suffer from are the scaling up complexity, the use of large quantities of volatile organic solvents, the heterogeneity of the liposomal product and the high cost of excipients as well as the lengthy multiple steps required.

2.3.2 *Novel techniques*

Many efforts have been made to improve the properties of liposome preparations, especially the stability, which is the major problem in the way of new advances. Presently, numerous novel methods have been successfully developed and seem to be more effective in producing narrowly sized vesicles in just one-step process and without need to use organic solvent for most of them.

2.3.2.1 *Freeze drying*

This novel method was first described by Li and Deng for the preparation of sterile and pyrogen-free narrow sizes liposomes; the method is based on the formation of homogenous dispersion of lipids in water-soluble carrier materials such as sucrose [36]. Lipids and sucrose are dissolved in cosolvent systems (*tert*-butyl alcohol/water) to form an isotropic monophase solution. The resulting solution is lyophilized by filtration after sterilization. The freeze-drying process was reported as follows: freezing step at -40°C for 8 h; primary drying at -40°C for 48 h and secondary drying at 25°C for 10 h [37]. The chamber pressure was maintained at 20 pascals during the drying process. The lyophilized product spontaneously forms homogenous submicron vesicles under aqueous hydration. Applying this preparation technique, both passive and active drugs loading within liposomes was possible [36]. However, this method results in low encapsulation efficiency unless it is combined with active loading [38].

2.3.2.2 *Heating method*

Mozafari proposed this method for fast production of liposomes without the use of any hazardous solvent and by simplifying the process [26]. The method involves the hydration of the lipids in an aqueous medium followed by a heating step at 120°C, in the presence of glycerol (3% v/v) [39,40]. Glycerol is a water-soluble, bioacceptable and non-toxic agent. Unlike the volatile organic solvents usually used in the conventional manufacturing techniques, glycerol is not removed from the final preparation; it can serves as an isotonic agent and as a dispersant and may prevent coagulation or sedimentation of the vesicles thereby enhancing the stability of the liposome suspensions [26].

Therefore, it is important to know that heating temperature at 120°C is required only when cholesterol or other sterols are used in the formulation [39]. Since the majority of phospholipids have their Tc below 60°C, and in the absence of other excipients requiring high temperatures to be dissolved, liposomes can be prepared at 60 – 70°C.

In conclusion, the heating method allows the sterilization of the liposome preparation and reduce the time and cost of liposome production [40]. Furthermore, resulted liposomes are also ready and ideal for freeze drying (e.g., in the preparation of dry powder inhalation products).

2.3.2.3 *Crossflow injection*

The liposome preparation technique based on the concept of continuous crossflow injection is an improvement to the traditional ethanol injection method in which a lipid/ethanol mixture is slowly injected into a highly sheared buffer solution [41,42]. The preparation system used by Wagner *et al.* consists of the crossflow injection module, vessels for the polar phase (phosphate buffered saline), an ethanol/lipid solution vessel and a nitrogen pressure device; the crossflow injection modules were made of two stainless steel tubes welded together to form a cross (**Figure 2**). At the connecting point, the modules are adapted with an injection hole drilled by spark erosion (150 and 250 mm drill holes) [41]. For liposomes production, the aqueous phase is pumped using a peristaltic pump through the crossflow injection module, the organic lipid solution is injected into the polar phase under nitrogen pressure. Process parameters such as the lipid concentration, the injection hole

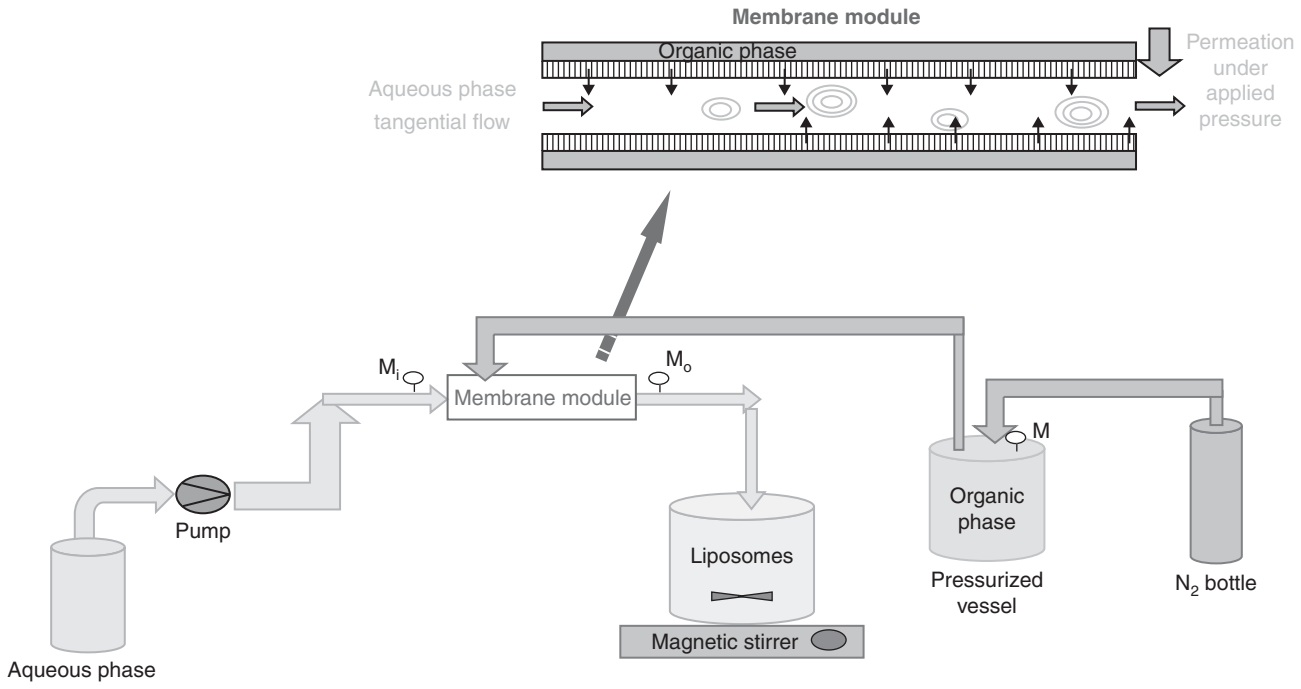

Figure 3. Schematic sketch of the membrane contactor experimental set up.
M: The pressurized vessel monometer; M, M_i and M_o: Monometers; M_i and M: Inlet and outlet monometers of the membrane device respectively.

diameter, the injection pressure, the buffer flow rate and system performance effect on liposome properties were investigated [41,42]. It was observed that, the injection hole diameter and the system performance do not influence the vesicle-forming process, but a minimum of a buffer flow rate is required to affect the batch homogeneity. By contrast, lipid concentration in combination with increasing injection pressures seems to be the strongly influencing parameters [41]. The resulting liposomes size using the crossflow injection method ranges between 200 and 500 nm.

Compared with the other technologies, in the crossflow injection method no mechanical forces are needed to generate homogeneous and narrow-sized liposomes. Another important advantage is the suitability for the entrapment of different drug classes such as large hydrophilic proteins by passive encapsulation, small amphiphilic drugs by a one step remote loading technique or membrane association of antigens for vaccination approaches [43].

2.3.2.4 *Microfluidic channel*

The controlled formation of submicrometer-sized liposomes using a microfluidic channel network was first reported by Jahn *et al.* [44].

Accordingly, a stream of lipids dissolved in alcohol is hydrodynamically sheathed between two oblique aqueous streams through a microfluidic channel (**Figure 3**). The lipid stream is focused on a very narrow sheet, with a thickness varying from a few micrometers down to submicrometers, depending on the respective volumetric flow rate ratios [45]. Thus, the laminar flow in the microchannel enables controlled diffusive mixing at the two liquid interfaces where the lipids self-assemble into vesicles [46]. By adjusting the ratio of the alcohol-to-aqueous volumetric flow rate, obtained vesicle are tunable over a mean size from 50 to 150 nm [46].

Process parameters investigations showed that liposome formation depends more strongly on the focused alcohol stream width and its diffusive mixing with the aqueous stream than on the sheer forces at the solvent–buffer interface. Hence, adjusting the flow parameters allows an effective way to modulate the average liposome size and size distributions obviating the need for post-processing [45].

2.3.2.5 *Membrane contactors*

This technique, firstly reported for the preparation of emulsion, polymeric and lipidic nanoparticles, was presently applied for liposomes preparation [47]. The term 'membrane contactor' can be defined as the connection of two phases A and B through the membrane pores (**Figure 4**). While applying a high pressure, the membrane contactor forces a dispersed phase A to permeate through the pores into a continuous phase B, which flows tangentially to the membrane surface [48]. According to published data, narrow-sized multilamellar liposomes having a satisfying entrapment efficiency were successfully prepared by the membrane contactor method [47,49]. Key advantages of this membrane based-process include the simple design, the easy control of vesicles size by tuning the process parameters and the ability to scale-up the production.

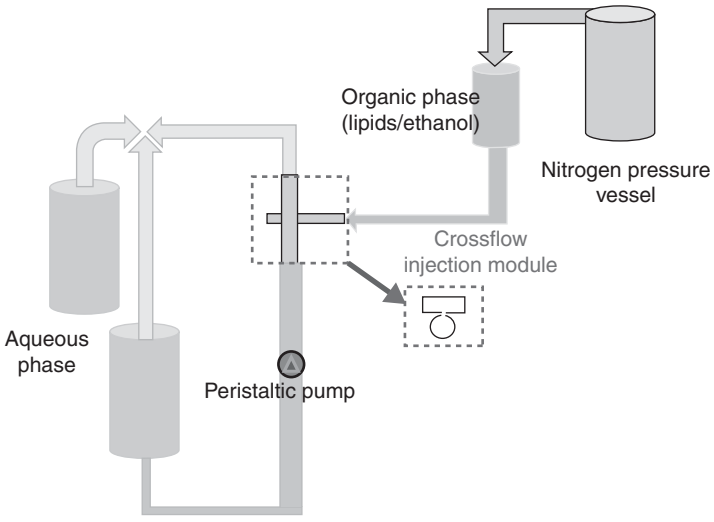

Figure 4. Crossflow injection module experimental set up as used by Wagner *et al.* to prepare liposomes [41].

2.3.2.6 CO_2 Supercrtitical fluid

Dense gas based-techniques offer the advantages of reducing the use of organic solvents, providing fast, single-stage production and producing stable and uniform liposomes [27]. The term dense gas is a general expression used to refer to a substance in the region surrounding the critical point; it possesses solvent power similar to that of liquids along with mass transport properties similar to those of gases. The most widely used dense gas is carbon dioxide since it is inflammable, non-toxic, non-corrosive, inexpensive, environmentally acceptable and has easily accessible critical parameters of 31.1°C and 73.8 bar [27].

Several dense gas technologies have been carried out on the formation of liposomes. The first dense gas techniques, referred as the injection and the decompression methods, were described by Castor and Chu in 1994. In 2005, Castor reported the production of small uniform liposomes using the super fluids phospholipid nanosome manufacturing process [50]. Frederiksen *et al.* described the supercritical liposome method in 1994, producing SUVs with particle size ranging between 20 and 50 nm [51]. Otake *et al.* developed the supercritical reverse phase evaporation (SCRPE) method [52]. Recently, the improved supercritical reverse phase evaporation (ISCRPE) technique was developed to enhance the stability and the drug loading efficiency [53].

3. Lipidic nanoparticles

3.1 Definition

SLNs have emerged as a novel approach to parenteral drug delivery systems. They are reported to be an alternative system and presenting further potential opportunities as they are combining the advantages of lipid emulsion systems, liposomes and polymeric nanoparticle systems while overcoming the temporal and *in vivo* stability issues that plague the aforementioned approaches.

SLNs are generally spherical in shape, in the submicron size range and are mainly composed of a solid lipid matrix stabilized by a surfactant interfacial region. Various ingredients such as triacylglycerol, fatty acids, acylglycerols, waxes and paraffins are usually used. Then phospholipids, sphingomyelins, bile salts such as sodium taurocholate, sterols such as cholesterol, are added as surfactant stabilizers. The biological nature of excipients leads to biocompatible and biodegradable carriers.

3.2 Preparation process

SLNs advantages included the ease to prepare and to handle and the stability at room temperature. Techniques commonly used to produce solid lipid nanoparticle are the HPH, the high-shear homogenization (HSH) and the microemulsion-based technique [54,55].

3.2.1 High-pressure homogenization

There are two general approaches within the HPH technique: the hot and the cold homogenization process [56]. For both techniques, the drug is dissolved or solubilized in the lipid being melted at approximately 5 – 10°C above its melting point.

For the hot homogenization technique, the drug-containing melt is dispersed under stirring in a hot aqueous surfactant solution at the same temperature [57]. Then the obtained pre-emulsion is homogenized using a piston-gap homogenizer, the resulted hot oil-in-water (o/w) nanoemulsion is cooled down to room temperature and the obtained lipids recrystallize forming the SLNs [58].

For the cold homogenization technique, the drug-containing lipid melt is cooled. The solid lipid ground to lipid microparticles (ranging from 50 to 100 µm) that are dispersed

in a cold surfactant solution yielding a pre-suspension [59]. The formed pre-suspension is homogenized, at or below room temperature, strong enough to break down the lipid microparticles and to form SLNs. This process avoids, or minimizes, the melting of the lipids and therefore minimizing loss of hydrophilic drugs to the water phase. The difference between the melting point of the lipid and the homogenization temperature needs to be large enough to avoid melting of the lipid in the homogenizer.

3.2.2 *High-shear homogenization*

HSH and ultrasound are widely used for producing SLNs [60]. These two dispersion techniques are widespread and easy to handle; they did not involve organic solvents or large amount of surfactants or any other additions as the HPH. However, these traditional techniques had the disadvantage of low dispersion quality as well as metallic contamination risks by using the ultrasound.

3.2.3 *Microemulsion*

Gasco developed an SLN preparation technique based on dilution in cold water of an o/w microemulsion [61]. For a microemulsion formulation, the lipid, solid at room temperature, must be heated above its melting point. Then surfactants and co-surfactants, including lecithin and bile salts, alcohols such as butanol, which is less favorable in regulatory terms, can be added. Subsequent addition of the microemulsion to water leads to precipitation of the lipid phase forming fine particles [61].

3.2.4 *Other techniques*

Other proposed techniques applied for SLN production include the solvent emulsification–evaporation or solvent emulsification–diffusion [62,63], solvent injection [64], membrane contactor [65] and supercritical fluid extraction of emulsions (SFEE), which is based on supercritical fluid extraction of the organic solvent from o/w emulsions [66].

4. Pulmonary applications

4.1 Liposomes

Almost from their rediscovery, liposomal vesicles have drawn attention of researchers as potential carriers of various therapeutic molecules that could be delivered by several routes. Liposomal formulations were commercialized in the 1980s as topical anti-aging products and later in 1995 – 1997 as intravenous delivery technologies of the liposome-based drugs: DaunoXome®, Doxil® and AmBisome®. The first pulmonary liposomal products in market include the synthetic lung surfactant Alveofact® (Biberach, Germany) for the treatment of respiratory distress syndrome.

The pulmonary delivery of liposomes has been explored for both systemic and local applications. The first principle aim of LBC is to improve bioavailability and enhance the release rate. The second is to reduce side effects and toxicity. Numerous studies, including *in vitro*, *ex vivo* and *in vivo* assessment, are illustrated with the data in **Table 1**. Nitrocamptothecin-loaded liposomes were effectively deposited in the human respiratory tract and were presenting sustained-release characteristics; the aerosol administration was found to be feasible and safe. Cyclosporin-liposomes directly deliver and accumulate the drug within lung. Aerosol administration of amphotericin B liposomal formulation could be an additional approach to optimizing treatment of invasive pulmonary aspergillosis. The importance of targeting potential of ligand-anchored liposomes has been established as rapid attainment of high-drug concentration in lungs with high population of alveolar macrophages was achieved and maintained over prolonged period of time.

Otherwise, encapsulating rifampicin and amikacin within liposomes decreases the toxic and side effects as well as the severity of damage to lungs following inhalation. Furthermore, liposomes inhalation was well tolerated and safe as no drug-related side effects were observed in most cases.

It can be concluded that investigated liposomal formulations (**Table 1**) have given promising results; reported works demonstrated that drugs delivered to the respiratory tract in liposomal formulation resulted in high pulmonary drug concentration, target effect, reduced systemic toxicity and reduced dosage requirements compared with parenteral and oral administration [64]. Some products successfully reached the clinical trials such as cisplatin (SLIT cisplatin, Transave, Inc., Monmouth Junction, NJ, USA) and fentanyl (AeroLEF, Delex Therapeutics, Mississauga, Ont., Canada).

4.2 Solid lipid nanoparticles

SLNs were investigated as a drug delivery system via several administration routes [67-70]. Being biodegradable systems, they served as alternatives to the usual colloidal carriers, particularly, polymeric nanoparticles.

Recently, the SLN aerosol offered a new and upcoming area of research and are proposed as carriers for pulmonary anticancer or peptide to improve their bioavailability [71]. The biodistribution of inhaled radiolabeled SLN has been described and the data showed an important and significant uptake into the lymphatics after inhalation [72]. A high rate of distribution in periaortic, axillar and inguinal lymph nodes was observed indicating that SLN could be effective colloidal carriers for lympho-scintigraphy or therapy on pulmonary delivery [72]. Furthermore, the originality of this colloidal system was to deliver the entrapped drug to the lung lymphatics through a pulmonary route which could be useful for cancer therapy.

Many drugs, for example, cyclosporine A [70], dexamethasone [73], budesonide [74], diazepam [75], paclitaxel [76], etc., and genes [77] have been successfully incorporated in SLNs for pulmonary drug delivery. Further examples of *in vitro* and *in vivo* evaluation studies carried out by different research groups on SLNs for pulmonary route are listed in **Table 2**. According to results, the advantages of SLNs are the achievement of a

Table 1. Recent examples of liposomes application developed for pulmonary administration: formulation and characterization.

Therapeutic class	Active principal ingredient	Objective and main results of the study	Ref.
Anticancer	Nitrocamptothecin	*9-Nitrocamptothecin-liposome (L-9NC) was nebulized to mice with subcutaneous xenografts of human cancers, with murine melanoma and with human osteosarcoma pulmonary metastases → subcutaneous tumor growth was greatly inhibited or tumors were undetectable after several weeks of treatment. It was also showed that oral dosage with L-9NC had no detectable effect on cancer growth, and thus the benefit from aerosol treatment was due to pulmonary deposition and not the larger fraction of drug deposited in the nose of mice during aerosol treatment, which is promptly swallowed → intramuscular L-9NC in slightly larger doses than given in the aerosol had detectable anticancer activity, but it was significantly less than in mice receiving the drug by aerosol	[82]
		*The efficacy of aerosol therapy using L-9NC was determined using two different experimental lung metastasis models **B16F10 murine melanoma cells → aerosol L-9NC treatment resulted in a reduction in lung weights and number of tumor foci. Visible tumor nodules were fewer and smaller in the treated group than in untreated control mice **SAOS-LM6 cell line derived from SAOS-2 human osteosarcoma → aerosol L-9NC was also effective against established lung metastases → it can be suggested that L-9NC aerosol therapy may offer significant advantage over existing methods in the treatment of melanoma and osteosarcoma pulmonary metastases	[83]
		**In vitro* release, *in vivo* mice tissue distribution and the damage to the lungs of L-9NC were investigated → after pulmonary delivery, the MRT of L-9NC in the lungs was 3.4 times as long as that of 9NC solution and the total AUC_{0-t} of all tissues in mice of the liposomes was 2.2-fold higher than that of the solution, indicating that the liposomes had sustained-release characteristics → the lung damage by liposomes was less severe than that by solution	[84]
		*The feasibility and safety of aerosol administration of the L-9NC formulation was evaluated. Patients with primary or metastatic lung cancer received aerosolized L-9NC for 5 consecutive days/week for 1, 2, 4 or 6 weeks followed by 2 weeks of rest to determine feasibility. For the Phase I part, the dose was increased stepwise from 6.7 up to 26.6 μg/kg/day Monday to Friday for 8 weeks followed by 2 weeks of rest → 9NC delivered as an aerosol was detected in patient's plasma shortly after the start of treatment	[85]
	Paclitaxel	*The pulmonary pharmacokinetics and therapeutic efficacy of paclitaxel liposomal formulations (L-PTX) administered by aerosol was evaluated. The therapeutic effect, deposition and clearance of L-PTX administered by aerosol or i.v. at comparative doses were performed on murine renal carcinoma (Renca) pulmonary metastases model → there was a significant reduction of the lung weights and the number of visible tumor foci on the lung surfaces of mice treated compared with control groups → inhalation of L-PTX also led to prolonged survival in mice inoculated with Renca cells	[86]

ACI: Andersen cascade impactor; AUC: Area under the curve; Chol: Cholesterol; DPI: Dry powder inhaler; DPPC: Dipalmitoylphosphatidylcholine; ECG: Electrocardiography; EE%: Encapsulation efficiency; FD: Freeze drying; L/D: Lipid-to-drug; MAC: *Mycobacterium avium* complex; MRT: Mean residence time; NE%: Nebulization efficiency; NER%: Retention of RIF after nebulization; OPM: *O*-palmitoyl mannan; OPP: *O*-polmitoyl pullulan; PC: Phosphatidylcholine.

Table 1. Recent examples of liposomes application developed for pulmonary administration: formulation and characterization (continued).

Therapeutic class	Active principal ingredient	Objective and main results of the study	Ref.
	Cyclosporin	*Comparative uptake of cyclosporine liposomal formulation (L-CsA) and propylene glycol solution (CsA-PG): the *in vitro* permeability of both formulations in a cell culture model (Calu-3 cells) was analyzed and the absorption process of CsA from human lung tissue into human blood *ex vivo* was elucidated → the permeability across the human bronchial cell line Calu-3 revealed low permeability for CsA with the apparent permeability for CsA-PG being twice as high as for L-CsA. The degree and rate of drug transfer into human blood was more pronounced for CsA-PG than for L-CsA with the AUC of CsA-PG being about 1.6 times higher than for the L-CsA formulation. The diffusion rate was more than 50% higher from CsA-PG than from the liposomes → both model systems consistently revealed that L-CsA displayed clearly a prolonged release effect and favorable longer tissue retention than CsA-PG	[87]
		*L-CsA was characterized *in vitro*, doses of 10 and 20 mg were inhaled with the PARI eFlow® inhaler by 12 stable lung transplant recipients, and lung deposition was evaluated by gamma scintigraphy → inhalation of CsA leads to lung deposition of 40 ± 6% (10 mg) and 33 ± 7% (20 mg), respectively. It resulted in a peripheral lung dose of 2.0 ± 0.4 mg (10 mg) and 3.4 ± 0.8 mg (20 mg), respectively. Extrathoracic deposition was 16 ± 6% (10 mg) and 14 ± 4% (20 mg), respectively, and the total deposition was calculated with 56% (10 mg) and 46% (20 mg). Lung deposition and peripheral lung deposition increased significantly with treatment time. Inhalation of the study medication was well tolerated, and led to only minor but statistically significant changes in lung function parameters *ex vivo*	[88]
Antibiotics	Amphotericin B	*The effects of treatment with aerosolized amphotericin B desoxycholate and aerosolized liposomal amphotericin B (L-AmpB) were evaluated in severely immunosuppressed rats with invasive pulmonary aspergillosis. The effects on pulmonary surfactant function were also evaluated *in vitro* → treatment with aerosolized L-AmpB significantly prolonged survival when compared with placebo-treated animals. AmpB desoxycholate inhibited surfactant function in a dose-dependent fashion, whereas, L-AmpB had no detrimental effect on surface activity of surfactant	[89]
		*Performance of L-AmpB for their selective presentation to lungs (alveolar macrophages), that being the densest site of aspergillosis infection, was studied. Egg PC- and Chol-based liposomes were modified by coating them with alveolar macrophage-specific ligands (OPM and OPP). The prepared formulations were characterized *in vitro* for vesicle morphology, mean vesicle size, vesicle size distribution and percent drug entrapment → *in vitro* airways penetration efficiency of the L-AmpB was determined by percent dose reaching the peripheral airways, it was recorded 1.4 – 1.6 times lower as compared with plain drug solution-based aerosol→ *in vivo* tissue distribution studies on albino rats suggested the preferential accumulation of OPM- and OPP-coated formulations in the lung macrophages. Higher lung drug concentration was recorded in case of ligand-anchored liposomal aerosols as compared with plain drug solution and plain liposome-based aerosols. The drug was estimated in the lung in high concentration even after 24 h. The drug-localization index calculated after 6 h was nearly 1.42-, 4.47- and 4.16-fold, respectively, for plain, OPM- and OPP-coated liposomal aerosols as compared with plain drug solution-based aerosols	[90]
	Rifampicin	*Rifampicin-loaded liposomes (L-RIF) were characterized *in vitro* for vesicle morphology, mean vesicle size and percent drug entrapment. *In vitro* airway penetration efficiency was determined by estimating percent dose	[91]

ACI: Andersen cascade impactor; AUC: Area under the curve; Chol: Cholesterol; DPI: Dry powder inhaler; DPPC: Dipalmitoylphosphatidylcholine; ECG: Electrocardiography; EE%: Encapsulation efficiency; FD: Freeze drying; L/D: Lipid-to-drug; MAC: *Mycobacterium avium* complex; MRT: Mean residence time; NE%: Nebulization efficiency; NER%: Retention of RIF after nebulization; OPM: *O*-palmitoyl mannan; OPP: *O*-polmitoyl pullulan; PC: Phosphatidylcholine.

Table 1. Recent examples of liposomes application developed for pulmonary administration: formulation and characterization (continued).

Therapeutic class	Active principal ingredient	Objective and main results of the study	Ref.
		reaching the base of the lung → results suggest the preferential accumulation of maleylated bovine serum albumin and *O*-steroyl amylopectin-coated formulations in the lung macrophages, which was reflected in the periodically monitored *in vivo* tissue distribution studies → the ligand-anchored liposomal aerosols are not only effective in rapid attainment of high-drug concentration in lung with high population of alveolar macrophages but also maintain the same over prolonged period of time. The significance of targeting potential of the developed systems was established	
		*L-RIF were characterized for EE%, size distribution, zeta-potential, stability during FD, NE% and NER% as well as mucoadhesion and toxicity in A549 cells → lower cytotoxicity of L-RIF compared with free drug	[92]
		*RIF-chitosan (CHT)-coated liposomes (L-CHT-RIF) were evaluated as well as their mucoadhesive properties and their cytotoxicity toward alveolar epithelial cells → highly improved mucoadhesive properties → lower cytotoxicity of L-RIF, which may be due to lower uptake by the cells, or slow release of RIF from liposomes	[93]
		*The *in vitro* intracellular activity of L-RIF against MAC residing in macrophage-like J774 cells was evaluated → liposomes were able to inhibit the growth of MAC in infected macrophages and to reach the lower airways in rats	[94]
	Amikacin	*Liposomal amikacin formulation (Arikace™), (Transave, Inc., Monmouth Junction, NJ, USA) that is being developed as an inhaled treatment for Gram-negative infections was aerosolized with an eFlow® (PARI, GmbH, Munich, Germany) nebulizer. Analysis for L/D (w/w) ratio, amikacin retention and liposome size were realized using an ACI → for the nebulized solution, 99.7% of the total deposited drug was found on ACI stages 0 through 5, which have cut-off diameters of 9, 5.8, 4.7, 3.3, 2.1 and 1.1 μm, respectively. Properties were found to differ for drug reclaimed on stage 0 compared with stages 1 – 5, which were not different from one another. For drug found on stages 1 – 5 (97% of total drug), the averages (n = 3) for L/D, percent encapsulated amikacin and liposome mean diameter ranged from 0.59 to 0.68 (w/w), 71 to 75%, 248 to 282 nm, respectively. Drug found on stage 0 (2.8% of total drug) had an average L/D ratio of 0.51 and average liposome mean diameter of 375 nm	[95]
		*Radiolabeled amikacin-liposomes(*L-AMK) were nebulized at a nominal dose of 120 mg for 20 min in healthy male volunteers. Lung deposition was determined by gamma scintigraphy following nebulization → total lung deposition, expressed as a percentage of the emitted dose, was 32.3 ± 3.4%. The time-dependent retention was biphasic with an initial rapid reduction in counts, followed by a slower phase to 48 h. The overall mean retention at 24 and 48 h was 60.4 and 38.3% of the initial dose deposited, respectively → the observed clearance of radiolabel is consistent with clearance of amikacin following aerosol delivery to rats. There were no clinically significant changes in laboratory parameters, vital signs or ECG. No adverse events including cough or bronchospasm were reported	[96]
Corticosteroids	Budésonide (DPI)	*Budesonide-liposomes (L-BUD) or drug-free BUD was delivered to rat lungs by intratracheal administration. The pulmonary disposition was assessed by monitoring the drug levels in the bronchoalveolar lavage and lung tissue → cumulative drug levels in the lung tissue indicated that the targeting factor was at least 1.66 times higher in liposomes; the maximal drug concentration in the lung homogenate was 36.64 mg as compared	[97]

ACI: Andersen cascade impactor; AUC: Area under the curve; Chol: Cholesterol; DPI: Dry powder inhaler; DPPC: Dipalmitoylphosphatidylcholine; ECG: Electrocardiography; EE%: Encapsulation efficiency; FD: Freeze drying; L/D: Lipid-to-drug; MAC: *Mycobacterium avium* complex; MRT: Mean residence time; NE%: Nebulization efficiency; NER%: Retention of RIF after nebulization; OPM: *O*-palmitoyl mannan; OPP: *O*-polmitoyl pullulan; PC: Phosphatidylcholine.

Table 1. Recent examples of liposomes application developed for pulmonary administration: formulation and characterization (continued).

Therapeutic class	Active principal ingredient	Objective and main results of the study	Ref.
		with 78.56 mg with the free BUD → the time for maximum drug concentration in the lung homogenate for the L-BUD was 9 – 12 h as compared with 3 h for that of free BUD	
		*Comparison of weekly BUD-stealth-liposomes therapy to daily free BUD therapy in reducing allergic inflammation → L-BUD is as effective as daily budesonide in reducing markers of lung inflammation in experimental asthma which may offers an effective alternative to standard daily BUD therapy in asthma and has the potential to reduce toxicity and improve compliance	[98]
Peptides	Insulin	*The encapsulation efficiency, particle size, lung disposition and the effect of reducing glucose concentration of the novel nebulizer-compatible liposomal carrier of insulin were investigated for aerosol pulmonary drug delivery. The *in vivo* bioavailability of the insulin-encapsulated liposomes (L-INS) was verified on animal after direct inhalation of into the lungs → liposomal carriers were homogeneously distributed within the lung alveoli. L-INS promotes an increase in the drug retention-time in the lungs and more importantly, a reduction in extrapulmonary side effects, which results in enhanced therapeutic efficacies → alveolar lavage fluid evaluation showed that inhalation of aerosolized liposomal insulin in mice caused neither virulent effect nor inflammation or immunoreaction on the lungs	[99]
		The *in vivo* study of intratracheal instillation of L-INS to diabetic rats showed successful hypoglycemic effect with low blood glucose level and long-lasting period. The relative pharmacological bioavailability was as high as 38.38% in the group of 8 IU/kg dosages	[100]
	Isoniazid	*Isoniazid-entrapped liposomes (L-INH) were developed and evaluated for size, drug entrapment, release, *in vitro* alveolar deposition, biocompatibility, antimycobacterial activity and pulmonary surfactant action → L-INH were about 750 nm in diameter and had entrapment efficiency of 36.7 ± 1.8%. Sustained release of INH from liposomes was observed over 24 h → *in vitro* alveolar deposition efficiency exhibited about 25 – 27% INH deposition in the alveolar chamber on 1 min nebulization using a jet nebulizer. At 37°C, the formulation had better pulmonary surfactant function with quicker reduction of surface tension on adsorption (36.7 ± 0.4 mN/m) than DPPC liposomes (44.7 ± 0.6 mN/m) and 87% airway patency was exhibited by the formulation in a capillary surfactometer. The formulation was biocompatible and had antimycobacterial activity → L-INH could fulfill the dual purpose of pulmonary drug delivery and alveolar stabilization due to anti-atelectatic effect of the surfactant action, which can improve the reach of antitubercular drug INH to the alveoli	[101]

ACI: Andersen cascade impactor; AUC: Area under the curve; Chol: Cholesterol; DPI: Dry powder inhaler; DPPC: Dipalmitoylphosphatidylcholine; ECG: Electrocardiography; EE%: Encapsulation efficiency; FD: Freeze drying; L/D: Lipid-to-drug; MAC: *Mycobacterium avium* complex; MRT: Mean residence time; NE%: Nebulization efficiency; NER%: Retention of RIF after nebulization; OPM: *O*-palmitoyl mannan; OPP: *O*-polmitoyl pullulan; PC: Phosphatidylcholine.

Table 2. Recent examples of SLNs application developed for pulmonary administration: formulation and characterization.

Active principal ingredient	Preparation method	Objective and main results of the study	Ref.
Rifampicin, isoniazid and pyrazinamide	Emulsion solvent diffusion	*The chemotherapeutic potential of nebulized SLPs incorporating rifampicin, isoniazid and pyrazinamide against experimental tuberculosis was evaluated. For the majority (35.8 ± 3.2%) of nebulized SLPs: the size ranging from 1.1 to 2.1 μm, with an MMAD of 1.7 ± 0.1 μm and GSD of 1.9 ± 0.1 μm were suitable for bronchoalveolar drug delivery. Following a single nebulization of drug-loaded SLPs to guinea pigs, therapeutic drug concentrations were maintained in the plasma for 5 days and in the organs (lungs, liver and spleen) for 7 days whereas free drugs were cleared by 1–2 days. The mean residence time was increased by 14- to 21-fold, 7- to 8-fold and 13- to 21-fold compared with i.v., oral or nebulized free drugs, respectively. The relative bioavailability was increased 10- to 16-fold, whereas, the absolute bioavailability was increased by 8- to 10-fold. On infected guinea pigs, no tubercle bacilli could be detected in the lungs/spleen after 7 doses of nebulized drug-loaded SLPs treatment whereas 46 daily doses of orally administered drugs were required to obtain an equivalent therapeutic benefit. No alterations in serum bilirubin, ALT or ALP were observed in animals receiving drug-loaded or empty SLPs which indicated the safety of the formulation as far as biochemical hepatotoxicity is concerned	[102]
Insulin	Reverse micelle double emulsion	*Ins-SLNs for pulmonary drug delivery were developed; the formulation parameters effects on SLNs deposition properties were investigated. The size and PDI of original Ins-SLNs were 114.7 ± 2.1 nm and 0.105 ± 0.132. The size and PDI of Ins-SLNs deposited by Air-jet nebulization on various stages of a home-made twin-stage impinger (stages 0, 1 and 2) were 109.1 ± 2.0, 88.2 ± 1.8 and 97.8 ± 3.2 nm, 0.115 ± 0.232, 0.152 ± 0.113, 0.133 ± 0.158, respectively. No obvious aggregation, cleavage or collapses of Ins-SLNs were observed. **In vivo* pulmonary delivery of Ins-SLN was assessed by measuring blood glucose levels. Local fate of fluorescent-labeled Ins-SLNs in the alveolar region of rats was visualized by fluorescence microscopy after nebulization. Fasting plasma glucose level was reduced to 39.41% and insulin level was increased to approximately 170 μIU/ml 4 h after pulmonary administration of 20 IU/kg Ins-SLNs. A pharmacological bioavailability of 24.33% and a relative bioavailability of 22.33% were obtained using subcutaneous injection as a reference. FITC Ins-SLNs were effectively and homogeneously distributed in the lung alveoli. *The cytotoxic effects of the Ins, Ins-SLNs, free SLNs as well as surfactant used in the preparation procedure on the A549 cells were studied by the MTT assay. Following 72 h, the cell viability of A549 was over 95%. This result indicated that the SLNs had almost no or negligible cytotoxicity.	[103]
Epirubicin		*EPI-SLNs were prepared as an inhalable formulation for treatment of lung cancer. The physicochemical properties and *in vitro* pulmonary deposition of EPI-SLNs as well as pharmacokinetic profile in rats were studied. *In vitro* deposition study suggested that SLNs remained stable during nebulization with improved RF compared with EPI solutions. *In vivo* pharmacokinetic study showed that the drug concentration achieved by inhalation of EPI-SLNs was much higher than the drug concentration in plasma. Furthermore, the drug concentration in lungs after inhalation of EPI-SLNs was much higher than that after administration of EPI solutions. The cytotoxicity was tested in A549 alveolar epithelial cells and found to be not toxic in blank SLNs, however, the EPI-SLNs showed a higher cytotoxicity that remains equivalent to the toxicity of the EPI solution.	[104]

ALP: Alkaline phosphatase; ALT: Alanine transaminase; EPI-SLNs: Epirubicin-loaded solid lipid nanoparticles; FITC: Fluorescein isothiocyanate; GSD: Geometric standard deviation; Ins-SLNs: Insulin-solid lipid nanoparticles; MMAD: Mass median aerodynamic diameter; MBC: Minimum bacteriostatic concentration; MIC: Minimum inhibitory concentration; PDI: Polydispersity index; RF: Respirable fraction; SLPs: Solid lipid particles.

Table 2. Recent examples of SLNs application developed for pulmonary administration: formulation and characterization (continued).

Active principal ingredient	Preparation method	Objective and main results of the study	Ref.
Amikacin	Solvent diffusion homogenization technique	*Amikacin-loaded SLNs (AMK-SLN) were prepared; the size, zeta-potential, loading efficiency and release profile were studied. The MIC and MBC of AMK-SLN with respect to *Pseudomonas aeruginosa in vitro* were determined. AMK-SLN size and zeta-potential were 150 ± 4 nm and + 4 mV, respectively. These increased to 190 ± 7 nm and + 16 mV after freeze drying. The percentage of drug-loading efficiency was 87 ± 4%. Considering the release profile of AMK from the studied carrier, MIC and MBC could be about two times less in SLNs compared with the free drug. This more potent effect of AMK-SLN could be due to easier diffusion of SLN into the cellular membrane of *P. aeruginosa* cells.	[105]

ALP: Alkaline phosphatase; ALT: Alanine transaminase; EPI-SLNs: Epirubicin-loaded solid lipid nanoparticles; FITC: Fluorescein isothiocyanate; GSD: Geometric standard deviation; Ins-SLNs: Insulin-solid lipid nanoparticles; MMAD: Mass median aerodynamic diameter; MBC: Minimum bacteriostatic concentration; MIC: Minimum inhibitory concentration; PDI: Polydispersity index; RF: Respirable fraction; SLPs: Solid lipid particles.

prolonged release and the increase of the drug's therapeutic efficiency as can be shown with rifampicin, isoniazid and pyrazinamide against experimental tuberculosis. Furthermore, SLN pulmonary administration provided a targeted administration, which is expected to avoid high concentration of the drug at non-target tissues and reducing toxicity; this was observed with epirubicin entrapment within SLNs.

Although SLNs for the pulmonary delivery is not fully appreciated, considering the limited number of scientific paper about this carrier, toxicological profile of SLNs, is expected to be better than that of polymer-based systems, for example. On the other hand, stability under nebulization could be a main advantage compared with liposomes.

4.3 Dry powder formulations

Typically, liposomes-based formulations for pulmonary drug delivery have been delivered by nebulization. However, concerns arise from drug stability and leakage perspectives when nebulizers are used; to circumvent these issues, dry powder formulations have been developed [78]. In liposomal dry powder formulations, 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. The SFD process, used to manufacture a liposomal powder formulation, addresses many of the problems encountered by previous formulations and manufacturing methods [14]. Alternatively, liposomal dry powder formulations can also be formulated by supercritical fluid technologies.

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 [79].

In the case of SLNs, a dry powder inhalation system of insulin-loaded solid lipid nanoparticles (Ins-SLNs) was investigated for prolonged drug release, improved stability and effective inhalation [80]. Formulation was prepared by spray freeze drying of Ins-SLNs suspension, with optimized lyoprotectant. It has been shown that SLNs dry powder offer an increasing potential as an efficient and non-toxic lipophilic colloidal drug carrier for enhanced pulmonary delivery of insulin.

5. Conclusion

Pulmonary drug delivery of colloidal system is a non-invasive method that was developed to target specific site of action and/or sustaining the release of the therapeutics locally or systemically in the lung. LBCs have been found to be suitable for the administration of an increasing number of therapeutic molecules through the pulmonary route and have experienced exponential growth in the last few years.

Liposomes have been successfully used via inhalation; they exhibit a number of advantages in terms of amphiphilic

character, ease of production and biocompatibility rendering them a suitable candidate. SLNs, innovative therapeutic delivery system, offer a promising approach for the formulation of active substances via pulmonary route since they are combining the advantages of liposomes and polymeric nanoparticles while overcoming *in vivo* stability issues. SLNs have not been subjected to extensive research such as the case of liposomes, thus one can expect an increasing number of contributions for respiratory delivery in the future.

6. Expert opinion

LBCs, liposomes and SLNs, exhibit some well-defined and delicate characteristics, which have created an attractive and efficient approach for pulmonary delivery.

The major aim of such systems is to allow a more specific targeting of the drug and/or a controlled drug release, leading to improved therapeutic efficacy with reduced drug dosage and dosing frequency, limited side effects and best patient compliance. They offer better tolerance and superior safety over the existing formulation delivered via pulmonary route. Indeed, as given in **Tables 1** and **2**, lipid-based formulations of some drugs have shown a significant increase in therapeutic efficacy and a decrease in side effects incidence when tested on preclinical models or in humans, compared with their non-encapsulated formulations.

Otherwise, the challenge for drug delivery to the lungs is to understand the aerosol particles fate *in vivo* and their interactions with biological systems. It is important to keep in mind that once deposited, drug encounter a variety of biological barriers including mucus barriers and enzymes in the tracheobroncial region, and macrophages in the alveolar region. Molecules such as small peptides may be subject to enzymatic degradation, whereas larger particles that dissolve slowly are subject to phagocytosis by alveolar macrophages [81]. Furthermore, the residence time of an inhaled drug within the lungs depends on the site of deposition. In the conducting airways a significant proportion of the drug is entrapped in the mucus, however, at the surface of the alveoli, lung surfactant could enhance the solubility or limit the absorption of inhaled drug. Hence, further exploration and recent work on particle fate and persistence within the lungs is necessary to aid the design of improved formulations.

On the other hand, in addition to drug carriers, inhalation devices are equally important for effectiveness of pulmonary drug delivery as it could influence the aerodynamic parameters of inhaled particles and ultimately affect the site of aerosol deposition. Thus, the design of the appropriate delivery device and the optimal operating conditions (e.g., flow rate) are important.

Furthermore, it can be observed through literature that most published data were limited to *in vitro* and *in vivo* characterization and only few reports have been published concerning toxicological risks of inhaled lipidic carriers. Hence, the total innocuity of inhaled carriers is partially discussed and not fully evaluated. As clinical use of liposomes and SLNs requires toxicological risk assessment, new challenges are provided in conducting well-designed inhalation toxicology studies.

In short, it can be said that commercialization is impeded by the lack of the '*perfect colloidal system*' for inhalation. As a conclusion, a complete evaluation of technical, economic, therapeutic and toxicological aspects of LBCs delivered via the pulmonary route for local or systemic action is required to appreciate the real value of such system.

Declaration of interest

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

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