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Cu, Zn- Superoxide dismutase liposomal dry powder formulations production using supercritical CO_2-assisted spray-drying: A proof-of-concept

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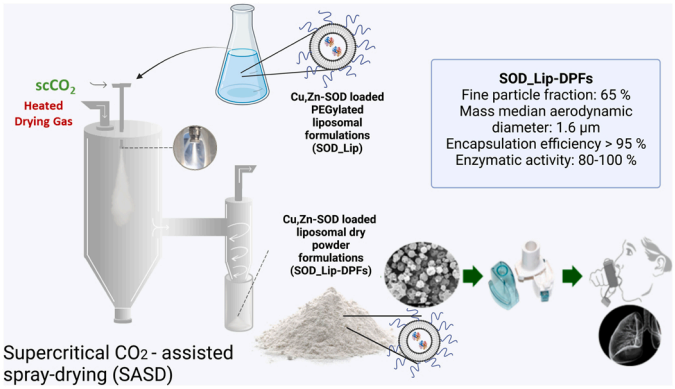
HIGHLIGHTS

- An inhalable enzyme formulation was produced using $scCO_2$-assisted spray drying.
- Trehalose proved to be effective and fundamental in liposome stability upon drying.
- Dried liposomes retained the enzyme structure, stability, and encapsulation efficiency.
- Dry powder formulations were stable for 50 days at 40% relative humidity.

GRAPHICAL ABSTRACT



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ABSTRACT

Enzyme-based inhalable therapeutics for lung inflammation are gaining interest as an alternative to long-term corticosteroids treatments. However, enzymes have poor pharmacokinetics. Encapsulating enzymes in liposomes can increase their half-live and modify their biodistribution. But both liposomes and enzymes are susceptible to destabilization during storage. This drawback can be surpassed, by converting liposomal suspension into solid dosage forms for different administration routes, including inhalation. In this study, Cu, Zn- superoxide dismutase (SOD) was encapsulated in liposomes, then dried using supercritical CO_2-assisted spray-drying to make SOD-loaded liposomal dry powder formulations (SOD_Lip-DPFs). Upon resuspension in water, liposomes maintained structural integrity, with 99% SOD encapsulation efficiency and preserved enzymatic activity. Stability studies showed that SOD_Lip-DPFs maintained liposomal and enzyme stability for 50 days at 40% relative humidity. This offers a stable and efficient delivery system for enzyme-based inhalable therapeutics.

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1. Introduction

Therapeutic proteins and particularly enzymes have aroused interest for the treatment of chronic inflammatory diseases. Global protein therapeutics are predicted to reach a value of US$ 456.0 billion by 2027 [1]. However, therapeutic outputs face limitations including low physical and chemical stability, protein degradation and immunogenicity [2]. Different non-invasive administration routes for enzyme delivery have been studied to overcome those disadvantages. In particular, pulmonary administration of enzymes has gained attention, due to the lung characteristics namely the thin blood-barrier membrane (0.1–0.2 μm), large absorption area (> 100 m^2) from a highly vascularized alveolar epithelium constituted by a single layer of cells, elevated blood flow (5 L/min) [3,4], and bypass the hepatic first-pass of metabolism. Other positive properties include the fact that the physiological pH and reduced mucociliary clearance in the deep lungs avoid rapid clearance, poor absorption, and exposure to digestive enzymes [5]. Inhalable protein-based therapies have attracted attention as the range of biological candidates for treating a wide range of respiratory diseases has broadened [6]. Ibrahim et al. [7] developed a spray-dried inhalable powder formulation of basic fibroblast growth factor (bFGF) for treating asthma or chronic obstructive pulmonary disease. However, powders with a fine particle fraction (FPF) of 25.2% resulted in limited delivery to the respiratory region. Wahjudi et al. [8] studied a formulation of spray-freeze dried acyl–homoserine lactone (AHL) acylase, using mannitol, trehalose, and inulin as excipients. The enzyme maintained its activity following drying. More recently, Fernandes et al. [9,10] reported the encapsulation of Cu, Zn- superoxide dismutase (SOD) in trehalose and leucine through spray-drying (SD). This work successfully addressed the impact of SD parameters on the aerodynamic performance, enzyme activity retention (EAR) and conformational stability SOD. SOD is an enzyme with a molecular weight of 32.5 kDa that catalyzes the dismutation of anion superoxide radicals in molecular oxygen and hydrogen peroxide. This enzyme is used in broad disease models, such as inflammation [11,12], rheumatoid arthritis [13,14], and ischemia-reperfusion injury [15,16]. In its free form and after IV administration, SOD has a bloodstream half-life of approximately 6 min in rats and 25 min in humans [17,18], essentially due to its rapid renal filtration. Enzyme encapsulation in liposomes – a DDS (drug delivery system) known by its biocompatibility, non-toxicity, and ability to encapsulate and carry hydrophilic molecules in its inner aqueous core [19] – might be advantageous insofar as liposomes not only protect enzymes from degradation and but also act as targeted vehicles, delivering them into the inflamed tissues [20]. Corvo et al. [21–23] have demonstrated improvement of the enzyme biodistribution and its therapeutic effect in inflammatory processes, increasing its half-life up to 20 h when SOD is encapsulated into PEGylated liposomes (liposomes with modified surface with polyethylene glycol to improve the liposomal suspension stability, prolong circulation time and avoid liposomes' opsonization [21–23]). This research showed that small-sized (110 nm) SOD-loaded liposomes were preferable for targeting SOD to the arthritic lesions in rats with adjuvant arthritis [23].

Enzyme-loaded liposomes can be converted into dry powder formulations (Lip-DPFs) to ensure their stability during storage. This avoids the need to cold distribution chains and facilitates access to the procedure by irrespective of location or social class. Over recent years, several techniques like freeze-drying [24,25], spray-freeze drying [6, 26], spray-drying [27,28] and supercritical fluids [27] have been proposed to produce Lip-DPFs. In a previous work, Costa et al. [27] used supercritical CO_2-assisted spray-drying (SASD) to convert PEGylated liposomes encapsulating a model hydrophilic dye molecule, (5(6)- carboxyfluorescein- CF) into Lip-DPFs. Different critical process parameters influenced certain critical quality attributes, such as size, polydispersity index (PdI), and encapsulation efficiency, which were evaluated using quality-by-design (QbD). After drying, liposomes maintained their structure and integrity, while the dye remained encapsulated in the inner aqueous phase of liposomes. The CF-loaded liposomal dry powder formulations also remained stable for 30 days at relative humidities of 4% and 50%. Building upon prior research, we investigate the feasibility of producing dry powder formulations of liposomes loaded with Cu, Zn-superoxide dismutase (SOD_Lip-DPFs) using supercritical fluid-assisted spray drying (SASD). Our objective is to determine whether this technique can achieve sufficient aerodynamic performance for efficient pulmonary delivery, while preserving the encapsulation efficiency and enzymatic activity of the SOD enzyme, for the effective treatment of lung inflammatory diseases. We also aim to create a stable SOD-liposome dry powder, which offers an advantage over the supply and distribution chains typically required for transporting and storing biopharmaceutical-loaded lipid-based nanoparticles. To our knowledge, no prior studies have investigated liposomal dry powder formulations encapsulating enzymes, particularly SOD, using SASD. Thus, this innovative work seeks to establish a robust, scalable, and time-efficient approach to producing an environmentally and patient-friendly inhalable protein delivery system.

2. Materials and methods

2.1. Materials

Egg-phosphatidylcholine (E-PC) (> 99%) and distearoylphosphatidylethanolamine-poly(ethyleneglycol)$_{2000}$ (DSPE-PEG_{2000}) (> 99%) were obtained from Lipoid GmbH (Ludwigshafen, Germany). Cholesterol (Chol) (> 99%), bovine erythrocytes Cu, Zn-superoxide dismutase, (SOD), citric acid monohydrate (> 99%) and L-leucine (98%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Trehalose dehydrated (> 98%) were acquired from Tokyo Chemical Industry (Chuo-ku, Tokyo), chloroform (> 99%) was purchased from Carlo Erba (Cornaredo, Milano, Italy) and ethanol absolute anhydrous (99.9%) was sourced from Scharlau (Barcelona, Spain). Air Liquide (Paris, France) provided carbon dioxide (99.998%). All components were used as received without further purification.

2.2. SOD-loaded liposomes preparation (SOD_Lip)

The liposomes were prepared using film hydration followed by extrusion methods [22]. Briefly, E-PC, Chol and DSPE-PEG_{2000} (molar ratio of 1.85:1:0.15, respectively) were dissolved in chloroform. The mixture was then dried using a rotary evaporator (Rotavapor RE-111-Buchi, Lugo, Swiss) to form a lipidic film. The obtained film was hydrated with a 10 mL of SOD, at a concentration of 0.5 mg/mL in a buffer solution (10 mM citric acid in 280 mM trehalose, at pH 6) at a total lipid concentration of 32 μmol/mL. The suspension rested for 1 h (SOD_Lip) at 19–21 °C, occasionally shaken. The liposomal suspension was extruded sequentially through membranes with pore sizes of 600, 400 and 200 nm using a high-pressure extruder (Lipex TM Thermobarrel Extruder, Biomembranes Inc., Vancouver, BC, Canada) with a Nucleopore® Track-Etched Membranes (Whatman®, Cytiva, Buckinghamshire, UK). Finally, the suspension was extruded three times through a membrane filter of 100 nm. Then, non-encapsulated SOD was removed by dilution and centrifugation using an Optima TM XL-90 Ultracentrifuge (Beckman Coulter, Carlsbad, CA, USA), for 2 h at 300,000 *g* and at 4 °C. The supernatant was then discarded, and the pellets were resuspended at the same concentration in the same buffer solution (10 mM citric acid and 280 mM trehalose, at pH 6). The liposomal formulations were sterilized by filtration through sterile filter with a pore size of 0.2 μm for SASD.

2.3. Dry powder liposomal formulations (Lip-DPFs)

For each assay, a 4% (v/v) suspension of liposomes was combined with an excipient solution consisting of trehalose and L-Leucine in a 9:1 (w/w) ratio and a mixture of water and ethanol in an 85:15 (v/v) ratio, with a total concentration of 300 mM. The resulting solution, which

contained the liposome suspension and all excipients, was referred to as the casting solution.

In each assay, the casting solution was fed into a laboratory scale SASD apparatus which was previously described in detail elsewhere [28]. Firstly, the CO_2 was liquified at − 5 ºC (at 25 mL/min) by passing the feeding line of carbon dioxide in a cryogenic bath which was then pumped using a high-pressure pump (HPLC pump K-501, Knauer, Berlin, Germany). The high-pressure CO_2 is then warmed using a heated bath (T_{CO2} = 80 ºC) before being delivered to the static mixer. Simultaneously, the casting solution, containing the SOD-loaded liposomes and the excipients, was loaded through a high-pressure pump (Smartline pump 1000, Knauer, Berlin, Germany), at 3.5 mL/min, into a heated static mixer, at 80 ºC, (3/16 model 37–03–075 Chemieer, Dayton, OH, USA) comprising a high-pressure column of 4.8 mm diameter, 191 mm length and 27 helical mixing elements that promoted CO_2 solubilization into the liquid solution, in order to obtain a near-equilibrium mixture (p_{SM} = 120 bar). The static mixer and the inlet streams were heated using tapes and a Shinko FCS-13A (Minoo, Osaka, Japan) temperature controller (± 0.2 ºC resolution). Afterwards, the mixture was atomized into the precipitator through a 150 μm internal diameter nozzle where the solvent underwent accelerated evaporation by heated compressed air flow ($F_{Air,in}$ = 30 m^3/h and $T_{Air,in}$= 100 ºC). The particles were separated from the CO_2-solvent flow in a high-efficiency cyclone and collected in a glass vessel.

The process yield is defined as follows:

$$\eta_{SASD}\ (\%) = \frac{mass\ of\ excipients\ after\ SASD(g)}{mass\ of\ excipients\ before\ SASD(g)} \times 100 \quad (1)$$

2.4. Reconstitution of SOD_Lip after SASD

After the SASD, a selected mass equivalent to 1 mL of liposomal formulation of SOD_Lip-DPFs was resuspended in deionized water to prevent osmotic shock to the liposomal formulations (final osmolarity of 300 mM). The excipients were removed by dilution and ultracentrifugation, for 2 h at 300,000 *g* and at 4 ºC. Then, the pellet was resuspended in 1 mL of the buffer solution (10 mM citric acid and 145 mM NaCl, at pH 6). The resulting SOD_Lip were characterized.

2.5. Liposome characterization

Before and after SASD, liposomes were characterized according to their average size and polydispersity index (PdI), as well as phospholipid and protein content to calculate SOD encapsulation efficiency (*EE*). A Zetasizer Nano S (Malvern Panalytical Ltd., Malvern, UK) was used to determine the mean particle size (Z-average), and the PdI, as a measure of the particle size distribution that ranged from 0 (monodisperse) and 1.0 (polydisperse). The concentration of phospholipids was determined by the Rouser's method [29]. Before SASD, the enzyme in liposomal suspension solution was quantified with a modified Lowry's method, where liposomes were previously disrupted with 2% (v/v) Triton® X-100 and 20% (v/v) of sodium dodecylsulphate (SDS) [21]. When needed and to avoid excipients interference in the protein quantification assay by the previously used Lowry's modified method, trehalose and leucine were previously removed by enzyme precipitation with trichloroacetic acid at 10% [10,11]. The SOD yield (η_{SOD}) was calculated as follows:

$$\eta_{SOD}\ (\%) = \frac{mass\ of\ SOD\ after\ SASD(\mu g)}{mass\ of\ SOD\ before\ SASD(\mu g)} \times 100 \quad (2)$$

The lipid yield (η_{Lipid}) was calculated as follows:

$$\eta_{lipid}\ (\%) = \frac{mol\ of\ lipids\ after\ SASD(\mu mol)}{mol\ of\ lipids\ before\ SASD(\mu mol)} \times 100 \quad (3)$$

Moreover, the percentage of encapsulation efficiency (*EE*) of SOD is given by the following equation:

$$EE\ (\%) = \frac{\left(\frac{\mu g_{SOD}}{\mu mol_{Lip}}\right)_f}{\left(\frac{\mu g_{SOD}}{\mu mol_{Lip}}\right)_i} \times 100 \quad (4)$$

where $\left(\frac{\mu g_{SOD}}{\mu mol_{Lip}}\right)_i$ is the initial SOD to lipid ratio and $\left(\frac{\mu g_{SOD}}{\mu mol_{Lip}}\right)_f$ is the final one.

The SOD enzymatic activity from SOD_Lip was evaluated using the SOD assay kit (19160 1KT-F, Sigma-Aldrich, St. Louis, MO, USA), where liposomes were previously disrupted with 2% (v/v) Triton® X-100 and 20% (v/v) of SDS. After 20 min, absorbance was measured at 450 nm in a microplate reader Infinite® 200 (Tecan Trading AG, Männedorf, Switzerland).

2.6. Characterization of liposomal dry powder formulations

2.6.1. Storage stability assays

The SOD_Lip-DPFs obtained from SASD were stored in triplicate in a desiccator at relative humidity (RH) of 40 ± 5% at 20 ºC. The RH was obtained using saturated solution of potassium carbonate. The temperature and the relative humidity were monitored using the Lasel® thermohygrometer data logger (Lascar electronics, Wiltshire, UK), model EL-USB-2, for 50 days, when the samples were characterized.

2.6.2. Morphology and particle size distribution

The SOD_Lip-DPFs' particle size and size distribution were determined using Morphologi G3 (Malvern, Paralab, Portugal) equipment. The width of the particle size distribution was evaluated by calculating the sample's span using the following equation:

$$span = \frac{Dv_{90} - Dv_{10}}{Dv_{50}} \quad (5)$$

where the Dv_{10}, Dv_{50} and Dv_{90} are the volume mean diameters respective to 10%, 50% and 90% of the sample population. Each sample characterization analyzed approximately 30,000 particles.

2.6.3. Scanning electron microscopy

The shape and morphology of SOD_Lip-DPFs particles were determined by scanning electron microscopy (SEM). The samples were put on a low vacuum sample holder, then a Hitachi S2400 (Osaka, Japan) analyzed the samples with an accelerating voltage set to 15 kV and at magnifications of 2000 x.

2.6.4. In vitro aerosolization study

The aerodynamic properties of the Lip-DPFs with and without SOD were determined using an eight-stage Andersen Cascade Impactor – ACI - (Copley Scientific, Copley Scientific, UK). After 5 (due to the static electricity) and 50 days, 30 mg of the powders were loaded into three hydroxypropylmethylcellulose capsules nº3 (Aerovaus, USA). The capsules were individually placed into a previously weighed dry powder inhaler (DPI) that was coupled to the ACI device. Each plate of the cascade impactor was covered by a pre-weighed filter (Glass Microfiber filter MFV1080, Filter Lab, Barcelona, Spain). The DPI punctured the capsule prior to the inhalation, and a high capacity pump was turned on to simulate an intake of breath: an air flow rate of 60 L/min lasting 4 s, according to the European pharmacopoeia [30]. The amount of powder deposited in each stage was calculated by weighing the filters before and after the test and calculating the difference. From this assay, several aerodynamic parameters were calculated—mass median aerodynamic diameter (MMAD), fine particle fraction (FPF), emitted dose (ED) and geometric standard deviation (GSD). The MMAD characterizes the size of those particles that reached the impactor, excluding those deposited in the throat. The value is displayed below, which is the diameter of 50% of the particles. On the other hand, FPF is the portion of the delivered particles sized below 5 μm, as determined by the interpolation of the

percentage of the particles with smaller sizes than this value. Finally, the GSD can be calculated using the following equation:

$$GSD = \sqrt{\frac{d_{84}}{d_{16}}} \quad (6)$$

where d_{84} and d_{16} are the diameters corresponding to 84% and 16% of the cumulative distribution, respectively. The emitted dose was calculated as follows:

$$ED = \frac{m_{full} - m_{empty}}{m_{powder}} \quad x100 \quad (7)$$

where m_{full} and m_{empty} are the weights (mg) of the capsule before and after simulating the inhalation and m_{powder} is the initial weight (mg) of the powder in the capsule.

2.6.5. Differential scanning calorimetry (DSC)

The glass transition temperature (*Tg*) was determined by weighing approximately 4 mg of powder on a TA Instruments differential scanning calorimeter (New Castle, DE, USA), DSC Q2000, within a temperature range from − 90–130.0 ℃ (10 ℃/min), in two cycles.

2.6.6. Residual ethanol content

Residual ethanol content was evaluated by high-performance liquid chromatography (HPLC) Dionex ICS3000 (Sunnyvale, CA, USA) with Pulsed Amperometric Detection (PAD). Briefly, Lip-DPFs with and without SOD were dissolved in Milli-Q water in a final concentration of 100 mg/mL. Then 10 μL of each sample was injected into a Thermo Carbopac PA10 250 × 4.6 mm with pre-column Thermo Aminotrap 50 × 4.6 mm (Waltham, Massachusetts, EUA). The analysis was conducted at a flowrate of 1 mL/min with NaOH (18 mM).

2.7. Statistical analysis

All the experiments were performed in triplicate as independent batches. All results are expressed as mean ± standard deviation (S.D.). To analyze the data, *t*-test with Welch's correction (GraphPadPrism, GraphPad software Inc., CA, USA) was used. The level of significance was set at the probabilities of * $p < 0.05$, and * * $p < 0.01$.

3. Results and discussion

3.1. Production of SOD-loaded liposomal dry powder formulations

Costa et al. [27], produced inhalable hydrophilic molecule-loaded liposomal dry powder formulations using the design-of-experiments approach to optimize the percentage of trehalose and leucine in the casting solution, so, after SASD, liposomes were able to keep their structure, while retaining the dye (5(6)-carboxyfluorescein) encapsulated. In this work, we hypothesized that the same optimized percentages of trehalose and leucine could produce SOD_Lip-DPFs. Empty and SOD-loaded liposomes with neutral charge were produced. A slight size increase was observed upon SOD encapsulation (Table 1). The initial SOD/Lip ratio (in the film hydration step) was approximately 15 μg/μmol. During extrusion, however, when passing through filters with several cut-offs, liposomes reorder themselves to decrease their size, leading to encapsulated enzyme loss in the inner core, resulting in a final SOD/Lip of 5 μg/μmol (Table 1). Following ultracentrifugation, empty liposomal and SOD-loaded liposomal formulations were sterilized and divided into two aliquots, one for storage at 4 ℃ and the other one to be dried using SASD equipment. Considering the critical process parameters (percentage of leucine (Leu) and ethanol) and the process variables optimized in the previous study, empty liposomes, and SOD_Lip were mixed with a casting solution (4%, v/v) composed of 90% (w/w) of trehalose (Tre) and 10% (w/w) of Leu (total solution osmolarity of 300 mM). The SASD experiment was carried out at $T\text{air}_{in} = 100 \pm 2$ ℃; $T_{CO2} = 80 \pm 2$ ℃; $T_{SM} = 84 \pm 4$ ℃; $T\text{air}_{out} = 64 \pm 1$ ℃; $p_{SM} = 120 \pm 2$ bar (three independent batches).

Table 1 shows a slight size decrease in the resuspended empty and loaded liposomes following drying. This might be related to two different phenomena. First, the water replacement theory suggests that during dehydration and in the solid state, the surrounding water molecules are replaced by hydrogen bonds with carbohydrates [31]. Second, as observed previously, leucine plays a role not only in moisture prevention but also in the maintenance of liposome structure. Electrostatic interaction between the carboxyl group of the Leu and the E-PC amine group might take place between leucine and liposomes, forming a layer on the liposome surface, capable of shielding them from external forces while drying [27]. Finally, the presence of ethanol in the casting solution can cause liposomes to shrink, resulting in a reduction of their size as studied by Pal et al. [32]. When compared to empty liposomes, the size decrease was more pronounced for SOD_Lip. It suggests possible interactions between the enzyme and the pegylated chain of DSPE--PEG_{2000} lipid. To confirm this assumption, further two-dimensional nuclear magnetic resonance studies must be performed [33]. Nonetheless, in our previous work [27] reconstituted CF-loaded liposomes from DPFs showed similar behavior, suggesting that the drying process and the casting solution composition might be the key critical process parameters to control the final liposome structure. Observations from Porras-Gómez et al. [34] showed that compression increase lipid alkyl chains disorder relative to the effect of cholesterol on gel (solid ordered) phases. In addition, Elizondo et al. [35] argue that compressed CO_2 provides a more homogeneous path for the supramolecular organization of the lipids in their membrane. Consequently, during drying, liposomes kept their homogeneity (PdI ≈0.2). The SOD yield of 99 ± 3% (with a respective *EE* over 95%), reveals that liposomes maintained their structure, retaining the enzyme inside aqueous the inner core. Otherwise, a significant decrease in the SOD yield would have been expected.

In addition to SASD enzyme yield, it is also worth considering SOD enzymatic activity retention. SOD activity was thereby assessed and percentages ranging from 80% and 100% were observed, proving that SASD does not significantly affect enzyme integrity and consequent

Table 1
Physicochemical characterization, encapsulation efficiency (*EE*) and retained enzymatic activity of liposomes and SOD_Lip, before and after SASD.

		Size [nm]	PdI	η_{Powder} [%]	η_{Lipid} [%]	η_{SOD} [%]	$[SOD/Lip]_f$ [μg/μmol]	*EE* [%]	Ret.Act. [%]
Before SASD	Empty liposomes	131 ± 1	0.048 ± 0.011	-	-	-	-	-	-
	SOD_Lip	136 ± 3	0.060 ± 0.007	-	-	-	5 ± 1	-	> 95
After SASD*	Empty liposomes	122 ± 3	0.174 ± 0.014	57 ± 6	58 ± 4	-	-	-	-
	SOD_Lip	117 ± 3	0.211 ± 0.009	65 ± 3	81 ± 2	99 ± 3	7 ± 1	> 95	80–100

*Liposomes which were resuspended from the powders
The results are expressed as mean ± S.D (n = 3).
PdI: Polydispersity index.
$[SOD/Lip]_f$: Final SOD to lipid ratio considering the final lipid concentration value

enzymatic activity. Lo et al. [36] produced spray-dried SOD-loaded liposomal formulations, in which the main phospholipid was DPPC and the excipient was sucrose. SOD enzymatic activity was preserved up to 98%, demonstrating the protective role of liposomes as drug delivery systems. Moreover, this work addresses the limitations of liposomal aerosols, as during nebulization, the applied shear stress may cause physical disruption of the liposome bilayers and loss of the entrapped drug. However, in our study, the liposomal dry powder formulation maintained both liposome integrity and enzymatic activity, indicating its potential as an effective and stable inhalable therapeutic [37].

Aerodynamic characterizations were then performed to verify if the SOD_Lip-DPFs were suitable for pulmonary delivery. Fig. 1. A shows the percentage of SOD_Lip-DPFs trapped on each Andersen Cascade Impactor (ACI) stage, where is possible to observe a higher percentage of powder trapped in stages 4 and 5. Therefore, SOD_Lip-DPFs presented an MMAD of 1.6 ± 0.4 μm and an FPF of 65 ± 5% (Table 2), the latter indicating the percentage of particles that achieve the lower respiratory tract (aerodynamic size < 5 μm), particularly the terminal bronchi. Table 2 also shows a volumetric diameter (Dv_{50}) of 4.6 μm with a respective span of 1.6, highlighting a narrow powder volumetric diameter distribution. These values are corroborated with SEM images (Fig. 1B), where it is possible to observe small well-defined spherical particles.

The emitted dose (Table 2) indicates that 98.6 ± 0.4% of the powder content can be fluidized by the airflow through the inhaler. Finally, HPLC analysis failed to detect ethanol peaks at powder concentrations up to 100 mg/mL. The regulated threshold for residual ethanol was 5000 ppm [38]. For this reason, SOD_Lip-DPFs can be administered through inhalation and are devoid of any residual organic contamination.

3.2. Stability assays of SOD_Lip-DPFs

Stability assays of the powders at a relative humidity of 40 ± 5% at 20 ℃, were conducted for 50 days. As a control, SOD_Lip suspensions were stored at 4 °C for an identical duration. After 50 days, the suspensions were resuspended in deionized water, and the excipients were removed via dilution and ultracentrifugation, with the discarded supernatant. The resulting liposome pellet was resuspended in buffer and characterized. Table 3 shows a non-significant increase in liposome size which was expected due to observations in DSC studies. Increased liposome size is often related to the modification of trehalose glass transition temperature. Sun et al. [39] suggest that an increase in molecular mobility with the decrease of trehalose glass transition temperature results in a liposome size increase. In this case, the T_g was not affected. DSC studies showed a T_g of 128 ℃ (the same before storage, data not shown). The *EE* remained above 95%, highlighting that despite the storage in such relative humidity, trehalose prevented drug leakage, reflecting its role as a stabilizing agent. Not only was *EE* maintained, but the SOD retained its enzymatic activity after 50 days storage. This demonstrates that, in terms of nanosystem, SOD_Lip-DPFs can prevent liposome fusion and rearrangement, keeping the enzyme in liposomes and protecting its enzymatic activity.

Table 2
Aerodynamic and residual solvent characterization of Lip-DPFs and SOD_Lip-DPFs.

	MMAD [μm]	GSD	Dv_{50} [μm]	span	FPF [%]	ED [%]	Residual ethanol [ppm]
Lip-DPFs	1.7 ± 0.1	2.3 ± 0.1	-	-	59 ± 7	98 ± 1	n.d.
SOD_Lip-DPFs	1.6 ± 0.4	2.3 ± 0.1	4.6	1.6	65 ± 5	98.6 ± 0.4	n.d.

The results are expressed as mean ± S.D (n = 3), excepting Dv_{50} and span (singular observation).
n.d.- Not detected.

Regarding the powders' aerodynamic properties (Fig. A.1, Supplementary data), after 50 days the MMAD increased from 1.6 ± 0.4 μm to 4 ± 1 μm ($p < 0.05$), while GSD presented a non-significant ($p > 0.05$) decrease to 1.9 ± 0.4, indicating a falling distribution of the aerodynamic diameter. FPF fell from 65 ± 5% to 28 ± 5% ($p < 0.01$), while the ED maintained its percentage ($p > 0.05$). The decrease in FPF indicates that only 28% of the particle managed to reach the respiratory region. Therefore, future research must be developed to further protect the powder from moisturizing and to improve the aerosolization efficiency of the stored powder. Despite the fall in powder flowability, drying has been shown to protect SOD-loaded liposomal formulations from external moisture. This eliminates the need for supply and distribution cold chains at temperatures of − 80 °C and − 20 °C, which pose a barrier to easy accessibility for individuals from diverse geographic and socio-economic backgrounds.

4. Conclusions

SOD was encapsulated into PEGylated liposomes and successfully dried in SASD, using trehalose and leucine as excipients. Considering previously optimized critical process parameters using a quality-by-

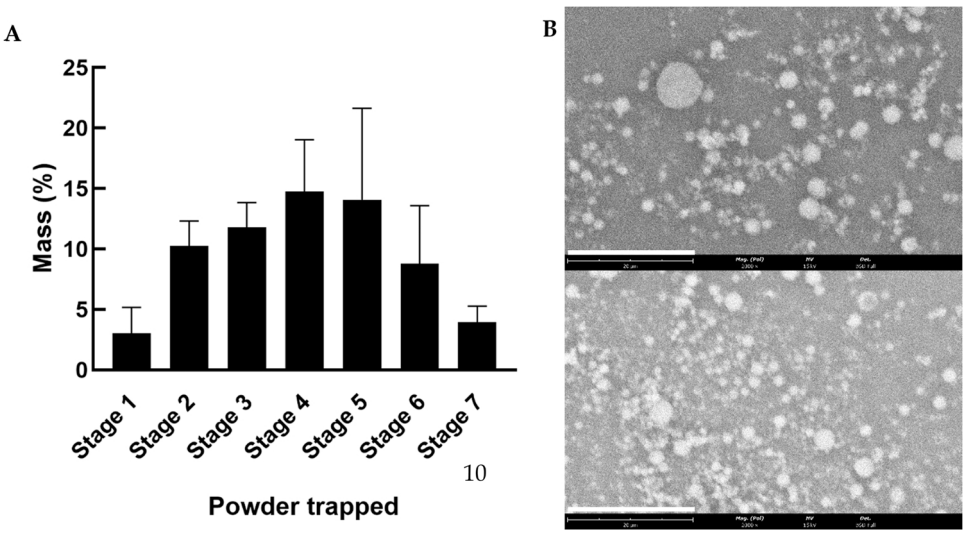

Fig. 1. - A) ACI tests for the powders obtained in the SASD. B) SEM images of SOD_Lip-DPFs (scale bar of 20 μm,15 kV with a magnitude of 2000 x.

Table 3
Physicochemical characterization, encapsulation efficiency (*EE*) and retained enzymatic activity (Ret.Act.) of SOD_Lip after 50 days at RH of 40 ± 5% at 20 ℃. SOD_Lip in suspension form was stored at 4 ℃ acting as control.

		Size [nm]	PdI	η_{Lipid} [%]	η_{SOD} [%]	[SOD/Lip] [μg/μmoL] $_f$	*EE* [%]	Ret.Act[¥] [%]
t = 0 days	SOD_Lip (suspension)	136 ± 3	0.060 ± 0.007	-	-	5 ± 1	-	> 95
	SOD_Lip From DPFs	117 ± 3	0.211 ± 0.009	81 ± 2	99 ± 3	7 ± 1	> 95	80–100
t = 50 days (RH= 50%)	SOD_Lip (suspension)	145 ± 5 ^{ns}	0.062 ± 0.020^{ns}	88 ± 1 ^{ns}	97 ± 5 ^{ns}	8 ± 1 *	> 95 ^{ns}	> 95 ^{ns}
	SOD_Lip From DPFs	124 ± 2 ^{ns}	0.185 ± 0.008 *	75 ± 6 ^{ns}	99 ± 9^{ns}	7 ± 1 ^{ns}	> 95 ^{ns}	90–100 ^{ns}

The results are expressed as mean ± S.D (n = 3).
[¥]n = 2 independent batches
ns: non-significant; * $p < 0.05$ compared to t = 0 days.

design, following drying, resuspended liposomes from dry powder formulations were revealed to have maintained both their structure and integrity, while preserving the enzymatic activity. Challenges regarding the chemical and physical stability of SOD-loaded liposomal formulations were surmounted by producing solid dosages form using the supercritical fluid drying method with optimized ratios of excipients. Moreover, the SOD_Lip-DPFs aerodynamic characterization proves that enzyme-loaded solid dosage form can reach the respiratory region, particularly the terminal bronchi. Stability studies showed that over 50 days, powders can protect liposomes and the encapsulated enzyme. However, decreased flowability suggests that more in-depth studies regarding stabilization agents (sugar) and moisture reduction (amino acid) must be carried out. Nonetheless, this work highlights the powder capacity to carry and protect enzyme-loaded liposomal formulations from moisture, with the same stability as those suspensions stored at 4 ℃, at least for 50 days, which represents a great advantage over the cold supply and distribution cold chains. Overall, this obviates the requirement for refrigeration-dependent supply chains and eases access to the formulations, regardless of geographic location.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.supflu.2023.105991.

References

[1] Protein Therapeutics Market Share, Size, Growth, Opportunity and Forecast 2022–2027, https://www.imarcgroup.com/protein-therapeutics-market (accessed January 16, 2023).
[2] S. Cao, S. Xu, H. Wang, Y. Ling, J. Dong, R. Xia, X. Sun, Nanoparticles: oral delivery for protein and peptide drugs, AAPS PharmSciTech 20 (2019) 190, https://doi.org/10.1208/s12249-019-1325-z.
[3] J.C. Sung, B.L. Pulliam, D.A. Edwards, Nanoparticles for drug delivery to the lungs, Trends Biotechnol. 25 (2007) 8, https://doi.org/10.1016/j.tibtech.2007.09.005.
[4] G. Pilcer, K. Amighi, Formulation strategy and use of excipients in pulmonary drug delivery, Int. J. Pharm. 392 (2010) 1–19, https://doi.org/10.1016/j.ijpharm.2010.03.017.
[5] B. Pulliam, J.C. Sung, D.A. Edwards, Design of nanoparticle-based dry powder pulmonary vaccines, Expert Opin. Drug Deliv. 4 (2007) 651–663, https://doi.org/10.1517/17425247.4.6.651.
[6] J.C.K. Lo, H.W. Pan, J.K.W. Lam, Inhalable protein powder prepared by spray-freeze-drying using hydroxypropyl-β-cyclodextrin as excipient, Pharmaceutics 13 (2021) 615, https://doi.org/10.3390/pharmaceutics13050615.
[7] B.M. Ibrahim, S.W. Jun, M.Y. Lee, S.H. Kang, Y. Yeo, Development of inhalable dry powder formulation of basic fibroblast growth factor, Int. J. Pharm. 385 (2010) 66–72, https://doi.org/10.1016/j.ijpharm.2009.10.029.
[8] M. Wahjudi, S. Murugappan, R. van Merkerk, A.C. Eissens, M.R. Visser, W.L. J. Hinrichs, W.J. Quax, Development of a dry, stable and inhalable acyl–homoserine–lactone–acylase powder formulation for the treatment of pulmonary Pseudomonas aeruginosa infections, Eur. J. Pharm. Sci. 48 (2013) 637–643, https://doi.org/10.1016/j.ejps.2012.12.015.
[9] D.A. Fernandes, E. Costa, P. Leandro, M.L. Corvo, Formulation of spray dried enzymes for dry powder inhalers: an integrated methodology, Int. J. Pharm. 615 (2022), 121492, https://doi.org/10.1016/j.ijpharm.2022.121492.
[10] D.A. Fernandes, P. Leandro, E. Costa, M.L. Corvo, Dry powder inhaler formulation of Cu,Zn-superoxide dismutase by spray drying: a proof-of-concept, Powder Technol. 389 (2021) 131–137, https://doi.org/10.1016/j.powtec.2021.05.008.
[11] C. Costa, Z. Liu, S.I. Simões, A. Correia, A. Rahikkala, J. Seitsonen, J. Ruokolainen, A. Aguiar-Ricardo, H.A. Santos, M.L. Corvo, One-step microfluidics production of enzyme-loaded liposomes for the treatment of inflammatory diseases, Colloids Surf. B 199 (2021), 111556, https://doi.org/10.1016/j.colsurfb.2020.111556.
[12] V.V. Shuvaev, R.Yu Kiseleva, E. Arguiri, C.H. Villa, S. Muro, M. Christofidou-Solomidou, R.V. Stan, V.R. Muzykantov, Targeting superoxide dismutase to endothelial caveolae profoundly alleviates inflammation caused by endotoxin, J. Control Release 272 (2018) 1–8, https://doi.org/10.1016/j.jconrel.2017.12.025.
[13] M.E.M. Cruz, M.M. Gaspar, M.B.F. Martins, M.L. Corvo, Liposomal Superoxide Dismutases and Their Use in the Treatment of Experimental Arthritis. Methods in Enzymology, Elsevier, 2005, pp. 395–413, https://doi.org/10.1016/S0076-6879(05)91022-7.
[14] M.L. Corvo, H.S. Marinho, P. Marcelino, R.M. Lopes, C.A. Vale, C.R. Marques, L.C. D. Martins, P. Laverman, G. Storm, M.B.A.F. Martins, Superoxide dismutase enzymosomes: carrier capacity optimization, in vivo behaviour and therapeutic activity, Pharm. Res 32 (2015) 91–102, https://doi.org/10.1007/s11095-014-1447-7.
[15] K. Tanaka, M. Shimoda, M. Kubota, A. Takafuji, M. Kawahara, T. Mizushima, Novel pharmacological effects of lecithinized superoxide dismutase on ischemia/reperfusion injury in the kidneys of mice, Life Sci. 288 (2022), 120164, https://doi.org/10.1016/j.lfs.2021.120164.
[16] M. Danková, I. Domoráková, Z. Fagová, M. Stebnický, A. Kunová, E. Mechírová, Bradykinin and noradrenaline preconditioning influences level of antioxidant enzymes SOD, CuZn-SOD, Mn-SOD and catalase in the white matter of spinal cord

in rabbits after ischemia/reperfusion, Eur. J. Histochem 63 (2019) 197–203, https://doi.org/10.4081/ejh.2019.3045.
[17] G. Jadot, A. Vaille, J. Maldonado, P. Vanelle, Clinical pharmacokinetics and delivery of bovine superoxide dismutase, Clin. Pharm. 28 (1995) 17–25, https://doi.org/10.2165/00003088-199528010-00003.
[18] M.L. Corvo, O.C. Boerman, W.J.G. Oyen, J.C.S. Jorge, M.E.M. Cruz, D.J. A. Crommelin, G. Storm, Subcutaneous administration of superoxide dismutase entrapped in long circulating liposomes: in vivo fate and therapeutic activity in an inflammation model, Pharm. Res 17 (2000) 600–606, https://doi.org/10.1016/s0005-2736(99)00081-4.
[19] G. Gregoriadis, Drug entrapment in liposomes, FEBS Lett. 36 (1973) 292–296, https://doi.org/10.1016/0014-5793(73)80394-1.
[20] M.E.M. Cruz, M.L. Corvo, M.B. Martins, S. Simões, M.M. Gaspar, Liposomes as tools to improve therapeutic enzyme performance, Pharmaceutics 14 (2022) 531, https://doi.org/10.3390/pharmaceutics14030531.
[21] M.L. Corvo, M.B. Martins, A.P. Francisco, J.G. Morais, M.E.M. Cruz, Liposomal formulations of Cu,Zn-superoxide dismutase: physico-chemical characterization and activity assessment in an inflammation model, J. Control Release 43 (1997) 1–8, https://doi.org/10.1016/S0168-3659(96)01473-3.
[22] M.L. Corvo, J.C.S. Jorge, R. Van't Hof, M.E.M. Cruz, D.J.A. Crommelin, G. Storm, Superoxide dismutase entrapped in long-circulating liposomes: Formulation design and therapeutic activity in rat adjuvant arthritis, Biochim. Et. Biophys. Acta - Biomembr. 1564 (2002) 227–236, https://doi.org/10.1016/S0005-2736(02)00457-1.
[23] M.L. Corvo, M.E.M. Cruz, D.J.A. Crommelin, G. Storm, Intravenous administration of superoxide dismutase entrapped in long circulating liposomes II. In vivo fate in a rat model of adjuvant arthritis, Biochim. Biophys. Acta - Biomembr. 1419 (1999) 325–334, https://doi.org/10.1016/s0005-2736(99)00081-4.
[24] D. Lu, A.J. Hickey, Liposomal dry powders as aerosols for pulmonary delivery of proteins, AAPS PharmSciTech 6 (2005) E641–E648, https://doi.org/10.1208/pt060480.
[25] J.D. Vanza, D.M. Shah, R.B. Patel, M.R. Patel, Afatinib liposomal dry powder inhaler: targeted pulmonary delivery of EGFR inhibitor for the management of lung cancer, J. Drug Deliv. Sci. Technol. 74 (2022), 103506, https://doi.org/10.1016/j.jddst.2022.103506.
[26] R. Bi, W. Shao, Q. Wang, N. Zhang, Spray-freeze-dried dry powder inhalation of insulin-loaded liposomes for enhanced pulmonary delivery, J. Drug Target 16 (2008) 639–648, https://doi.org/10.1080/10611860802201134.
[27] C. Costa, B. Nobre, A.S. Matos, A.S. Silva, T. Casimiro, M.L. Corvo, A. Aguiar-Ricardo, Inhalable hydrophilic molecule-loaded liposomal dry powder formulations using supercritical CO_2 – assisted spray-drying, J. CO_2 Util. 53 (2021), 101709, https://doi.org/10.1016/j.jcou.2021.101709.
[28] C. Moura, T. Casimiro, E. Costa, A. Aguiar-Ricardo, Optimization of supercritical CO_2-assisted spray drying technology for the production of inhalable composite particles using quality-by-design principles, Powder Technol. 357 (2019) 387–397, https://doi.org/10.1016/j.powtec.2019.08.090.
[29] G. Rouser, S. Fleischer, A. Yamamoto, Two-dimensional thin layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots, Lipids 5 (1970) 494–496, https://doi.org/10.1007/BF02531316.
[30] Council of Europe, Preparations for Inhalation: Aerodynaminc Assessment of Fine Particles, in: European Pharmacopeia, 5, 2010, pp. 274–285. https://file.wuxuwang.com/yaopinbz/EP7/EP7.0_01_235.pdf.
[31] P.T. Ingvarsson, M. Yang, H.M. Nielsen, J. Rantanen, C. Foged, Stabilization of liposomes during drying, Expert Expert Opin. Drug Deliv. 8 (2011) 375–388, https://doi.org/10.1517/17425247.2011.553219.
[32] A. Pal, P. Sunthar, D.V. Khakhar, Effects of ethanol addition on the size distribution of liposome suspensions in water, Ind. Eng. Chem. Res. 58 (2019) 7511–7519, https://doi.org/10.1021/acs.iecr.8b05028.
[33] C. Doyen, E. Larquet, P.-D. Coureux, O. Frances, F. Herman, S. Sablé, J.-P. Burnouf, C. Sizun, E. Lescop, Nuclear magnetic resonance spectroscopy: a multifaceted toolbox to probe structure, dynamics, interactions, and real-time *In Situ* release kinetics in peptide-liposome formulations, Mol. Pharm. 18 (2021) 2521–2539, https://doi.org/10.1021/acs.molpharmaceut.1c00037.
[34] M. Porras-Gómez, H. Kim, M.T. Dronadula, N. Kambar, C.J.B. Metellus, N.R. Aluru, A. van der Zande, C. Leal, Multiscale compression-induced restructuring of stacked lipid bilayers: from buckling delamination to molecular packing, PLoS ONE 17 (2022), e0275079, https://doi.org/10.1371/journal.pone.0275079.
[35] E. Elizondo, J. Larsen, N.S. Hatzakis, I. Cabrera, T. Bjørnholm, J. Veciana, D. Stamou, N. Ventosa, Influence of the preparation route on the supramolecular organization of lipids in a vesicular system, J. Am. Chem. Soc. 134 (2012) 1918–1921, https://doi.org/10.1021/ja2086678.
[36] Y. Lo, J. Tsai, J. Kuo, Liposomes and disaccharides as carriers in spray-dried powder formulations of superoxide dismutase, J. Control Release 94 (2004) 259–272, https://doi.org/10.1016/j.jconrel.2003.09.019.
[37] A. Elhissi, Liposomes for pulmonary drug delivery: the role of formulation and inhalation device design, Curr. Pharm. Des. 23 (2017) 362–372, https://doi.org/10.2174/1381612823666161116114732.
[38] Committee for Human Medicinal Products, ICH guideline Q3C (R6) on impurities: guideline for residual solvents (2017).
[39] W.Q. Sun, A.C. Leopold, L.M. Crowe, J.H. Crowe, Stability of dry liposomes in sugar glasses, Biophys. J. 70 (1996) 1769–1776, https://doi.org/10.1016/S0006-3495(96)79740-0.