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Optimization of quercetin - liposomal dry powders for pulmonary delivery using supercritical CO_2-assisted spray drying

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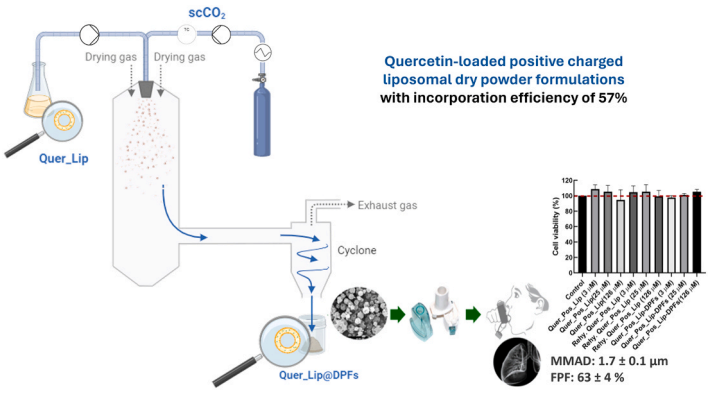
HIGHLIGHTS

- Quercetin-loaded liposomal dry powder formulations were prepared using SASD technology.
- Dried powders demonstrated suitable aerodynamic properties.
- Resuspended quercetin-loaded liposomes powder kept the liposomal properties

GRAPHICAL ABSTRACT



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ABSTRACT

Quercetin exhibits anti-inflammatory and antioxidant properties. Incorporating quercetin into liposomes can overcome its limited water solubility and poor oral bioavailability, making it a promising candidate for treating inflammatory diseases. For pulmonary administration, supercritical CO_2-assisted spray drying can be used to convert liposomal suspensions into dry powder formulations suitable for inhalation. However, the extraction power of $scCO_2$ can pose challenges on retaining the incorporation efficiency (*IE*) of this flavonoid in the lipid bilayer. This study focuses on optimizing quercetin's *IE* after drying using different liposomal lipid compositions with varying surface charges. The *IE* of quercetin into positively charged liposomes was 57 %. Additionally, the resulting powders had a mass median aerodynamic diameter of 1.7 µm and a fine particle fraction (particle size < 5 µm) of 63 %, indicating their suitability for inhalation. Cytotoxicity assays also revealed that both reconstituted liposomes and dry powder formulations were non-toxic to areolar fibroblast cells.

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1. Introduction

Chronic respiratory diseases, such as chronic obstructive pulmonary disease (COPD), asthma, and cystic fibrosis cannot yet be cured; however, long-term treatments involving inhaled corticosteroids can alleviate symptoms and enhance daily life. Unfortunately, prolonged therapy with inhaled corticosteroids may lead to various adverse side effects, including pneumonia [1–3], cataracts [2], and an increased risk of osteoporosis [2,4]. Quercetin (Quer), a polyphenolic flavonoid found in vegetables and fruits, exhibits remarkable anti-inflammatory and antioxidant properties [5]. It functions as an active oxygen scavenger and an estrogen receptor agonist. Studies by Mitani *et al.* [6] indicated that quercetin enhances anti-aging molecules, specifically Adenosine Monophosphate-Activated Protein Kinase (AMPK) activation and Nuclear Factor Erythroid 2-Related Factor 2 (Nrf2) expression. Additionally, it improves the corticosteroid responsiveness in COPD cells, suggesting its potential as a novel treatment for chronic respiratory diseases such as COPD. Furthermore, computational analyses by Derosa *et al.* [7] revealed quercetin's ability to impede SARS-CoV-19 replication. Despite its therapeutic benefits, quercetin has poor water solubility and low permeability, which significantly limits its therapeutic potential [8,9].

Liposomes, recognized for their biodegradability, biocompatibility, and non-toxicity, are promising drug delivery systems (DDS) [10,11]. Owing to their physicochemical attributes, liposomes can incorporate hydrophobic compounds in their bilayers, making them ideal candidates for carrying and delivering Quer. Numerous studies have assessed the antioxidant potential of Quer-loaded liposomes. Rezaei-Sadabady *et al.* [12] demonstrated the production of liposomal Quer formulations, which improved its solubility and bioavailability. Moreover, the cellular uptake of Quer is enhanced when encapsulated in liposomes. Ferreira-Silva *et al.* [13] investigated the efficacy of Quer liposomal nanoformulation, reporting an incorporation efficiency exceeding 95 %, effectively treating ischemia and reperfusion injury in a liver, for a rat model. Despite successful studies on the effect of Quer on inflammatory processes, chronic respiratory diseases predominantly affect the respiratory region. Therefore, a formulation designed for pulmonary drug delivery could offer increased efficacy and suitability for treating these diseases. Recently, Alhajj *et al.* [14] introduced a ciprofloxacin-Quer co-amorphous system for treating cystic fibrosis. Powders with a mass median aerodynamic diameter (MMAD) between 2.51 ± 0.05 and 3.5 ± 0.6 μm were obtained. However, due to the drugs' poor water solubility, 75 % ethanol was used during the process, resulting in powders with a high residual moisture content (2.95 ± 0.14–3.59 ± 0.13 %) exceeding the regulated threshold for ethanol [15]. Freeze and spray-dried solid dispersions comprising Quer, and polymers, such as polyvinylpyrrolidone, hydroxypropyl methylcellulose, poloxamer 400, and polyethylene glycol 8000, have also been explored to enhance Quer solubility [16,17]. Scalia *et al.* [18] developed solid lipid microparticles with a FPF of approximately 20 %. Papakyriakopoulou and colleagues [19] reported Quer-β-cyclodextrin derivative complexes with mannitol/lecithin microparticles for Alzheimer's disease treatment via nasal delivery. Supercritical fluid-based techniques, such Supercritical Fluid Extraction of Emulsion (SFEE) and Particles from Gas Saturated Solutions (PGSS), have also been used for producing solid dosage forms of Quer. For example, Lévai *et al.* [20] produced Quer suspensions using soybean lecithin and Pluronic L64® using SFEE, which were converted into microparticles using the PGSS-drying technology. The authors observed a significant improvement in Quer permeability through a transdermal membrane into the simulated intestinal fluid.

Previous studies have demonstrated that liposomal suspensions can be converted into liposomal dry powder formulations (Lip-DPFs) suitable for inhalation, with a MMAD between 1 and 5 μm and a fine particle fraction (FPF) above 60%, through supercritical CO_2-assisted spray-drying (SASD) [21]. SASD is a sustainable and environmentally friendly continuous process wherein supercritical CO_2 serves as a co-solute. Briefly, $scCO_2$ is solubilized into the liquid solution containing the drug and/or carrier system. The resulting near-equilibrium mixture outflowing from the saturator/static mixer is then atomized through a nozzle to the precipitation chamber at near atmospheric pressure [22]. This $scCO_2$-assisted drying process offers several advantages over conventional methods, including precise control over particle size and distribution by adjusting process parameters such as pressure and temperature [23,24]. Additionally, it allows operation at lower drying temperatures and with reduced organic solvent content, making it suitable for thermolabile compounds.

For example, Kasapoğlu *et al.* reported the conventional spray drying of liposomal formulations using an inlet temperature (T_{in}) of 150 ± 5 °C, resulting in an outlet temperature (T_{out}) of 85 ± 5 °C [25]. In contrast, Costa *et al.* [26] successfully produced enzyme-loaded Lip-DPFs through SASD with suitable aerodynamic properties for inhalation while preserving both liposome and enzyme integrity. They achieved this using a T_{in} of 100 ± 2 °C and T_{out} of 64 ± 1 °C, demonstrating the suitability of this process for thermosensitive compounds. However, optimizing SASD can be complex due to the numerous variables involved, including temperature, pressure, CO_2 and feed stream flow rates and the feed stream composition, all of which affect the final liposome, drug incorporation efficiency, and powder properties. To our knowledge, no study has yet investigated the production of Quer-loaded liposomal dry powder formulations (Quer_Lip-DPFs) using SASD. Therefore, this study aims to develop Quer_Lip-DPFs for inhalation purposes. However, since Quer is a flavonoid soluble in $scCO_2$, its stable incorporation into the lipid bilayer remains a challenge. To address this, our work primarily focuses on optimizing liposomal formulations to maximize the incorporation efficiency of Quer following powder dissolution. Additionally, the effects of varying leucine and ethanol concentrations on liposomal integrity were explored. Ultimately, this research aims to formulate patient-friendly, environmentally sustainable, and effective dry powder liposomal system for delivering flavonoids to treat lung inflammatory diseases.

2. Materials and methods

2.1. Materials

1,2-Dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1,2-dimyristoyl-sn-glycero-3-phosphorylglycerol sodium salt (DMPG), and egg-phosphatidylcholine (E-PC) were obtained from Lipoid (Germany). Cholesterol (Chol), stearylamine (SA) (99 %), L-leucine (98 %), citric acid and sodium chloride were purchased from Sigma-Aldrich (USA). Quercetin (>97 %) was purchased from Alfa Aesar (UK). Trehalose dehydrated (> 98 %) was acquired from Tokyo Chemical Industry (China), chloroform (> 99 %) was purchased from Carlo Erba (Spain) and ethanol absolute anhydrous (99.9 %) was sourced from Scharlau (Spain). α-Minimum essential medium (α-MEM), fetal bovine serum (FBS), penicillin, streptomycin and amphotericin B were purchased from Gibco ® (USA). Methanol (HPLC gradient grade) was purchased from Merck (Germany). Air Liquide (Portugal) provided carbon dioxide (99.998 %). All compounds were used as received without further purification.

2.2. Methods

2.2.1. Preparation of liposomes and quercetin-loaded liposomes

Liposomes were prepared using the film hydration method followed by extrusion [21]. Briefly, for neutral liposomes (*Neut_Lip*), a molar ratio of 9:1 (E-PC:Chol) was used. For positive-charged liposomes (*Pos_Lip*), a molar ratio of 8:1:1 (E-PC:Chol:SA) was employed. Finally, for negative liposomes (*Neg_Lip*), DMPC and DMPG were dissolved at a molar ratio of 7:3. All the formulations were prepared at a total lipid concentration of 32 μmol/mL and dissolved in chloroform in a round bottom flask.

For the preparation of Quer-loaded liposomes (*Quer_Neut_Lip*,

Quer_Neg_Lip, and *Quer_Pos_Lip*), in parallel Quer was first completely dissolved in methanol and then added to the lipid components dissolved in chloroform (molar ratio of 1:10). The mixtures were then dried using a rotary evaporator (Rotavapor RE-111- Buchi, Swiss), at 30℃, to form a lipidic film. The film was then hydrated with 10 mL of a buffer solution (10 mM citric acid in 280 mM trehalose, at pH 6), resting for 1 hour at room temperature, with occasional agitation. A high-pressure extruder (Lipex TM Thermobarrel Extruder, Biomembranes Inc., Canada) with a Nucleopore® Track-Etched Membranes (Whatman®, USA) with pore sizes of 600, 400 and 200 nm was sequentially applied to the obtained suspension and further extruded with three passes across a membrane filter of 100 nm. Finally, the non-incorporated Quer was separated from the liposomal formulation by size exclusion chromatography (SEC) using a desalting column with a 1000 Dalton cut-off (Econo-Pac®, BIO-RAD, USA). The produced liposomal formulations were sterilized by filtration through syringe 0.2 μm PTFE sterile filters and stored at 4°C, for further processing in SASD and/or characterization.

The percentage of incorporation efficiency (*IE*) of the Quer, after SEC, is given by the following equation:

$$I.E.(\%) = \frac{\left(\frac{\mu g_{Quer}}{\mu mol_{Lip}}\right)_f}{\left(\frac{\mu g_{Quer}}{\mu mol_{Lip}}\right)_i} \times 100 \quad (1)$$

where $\left(\frac{\mu g_{Quer}}{\mu mol_{Lip}}\right)_i$ is the initial Quer-to-lipid ratio (before extrusion) and $\left(\frac{\mu g_{Quer}}{\mu mol_{Lip}}\right)_f$ is the final ratio after SEC/before SASD.

2.2.2. Preparation of quercetin-loaded dry powder liposomal formulations

In each assay, 4 % (v/v) of the liposomal suspensions were mixed with a solution at a total concentration of 300 mM of trehalose (Tre) and 10 % (w/w) of leucine (Leu). Each suspension was prepared as a mixture of water:ethanol (85:15, % v/v). The final suspension containing the liposomes and all the excipients (Tre and Leu) is referred to as the feed stream from now on. In each assay, the feed stream was fed into a laboratory-scale SASD apparatus which was previously described in detail elsewhere [22]. First, the CO_2 was liquified (at 25 mL/min) by passing the feeding line of CO_2 in a cryogenic bath which was then pumped using a high-pressure pump (HPLC pump K-501, Knauer, Germany). The high-pressure CO_2 was then warmed using a heated bath (T_{CO2} = 80°C) before being delivered to the static mixer. Simultaneously, the feed stream, containing the empty liposomes or *Quer_Neut_Lip*, *Quer_Neg_Lip*, or *Quer_Pos_Lip*, along with the excipients (Tre and Leu), was pumped through a high-pressure pump (Smartline pump 1000, Knauer, Germany), at 3.5 mL/min, into a heated static mixer (3/16 model 37–03–075 Chemieer), set at 80 °C. The mixer featured a high-pressure column of 4.8 mm diameter, 191 mm length and 27 helical mixing elements, which promoted CO_2 solubilization into the liquid phase, resulting in a near-equilibrium mixture (p_{SM} = 120 bar). The static mixer and the inlet streams were heated using tapes and a Shinko FCS-13A temperature controller (± 0.2°C 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_{in}= 100 °C). 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:

$$\eta_{SASD}(\%) = \frac{amount\ of\ excipients\ after\ SASD\ (g)}{amount\ of\ excipients\ before\ SASD\ (g)} \times 100 \quad (2)$$

2.2.3. Reconstitution of liposomes after SASD

After the SASD, for resuspendability test characterization, a defined mass of liposomal formulations of empty liposomes, *Quer_Neut_Lip*, *Quer_Neg_Lip*, or *Quer_Pos_Lip* was resuspended in deionized water at a final osmolarity of 300 mM (to prevent an osmotic shock to the liposomal formulations). The excipient was removed by dilution and ultracentrifugation for 2 h at 300,000 xg, at 15℃. The obtained liposomal pellet was resuspended in buffer solution (10 mM citric acid in 140 mM NaCl, at pH 5.6). The resulting liposomes were then characterized.

2.2.4. Characterization of liposomal formulations

Before and after SASD processing, liposomes were characterized according to their mean size (Z-average) and polydispersity index (PdI), as well as phospholipid and Quer content used to calculate the incorporation efficiency (*IE*). A Zetasizer Nano S (Malvern Panalytical Ltd., Malvern, UK) determined Z-average, and the PdI as a measure of the particle size distribution that ranged from 0 (monodisperse) and 1.0 (polydisperse). The Rouser method [27] assessed the phospholipid quantification. Quer quantification was performed by high-performance liquid chromatography (HPLC) in a Purospher® STAR RP-18 end-capped (5 μm) HPLC column, 4.6 × 250 mm (Merck, Germany), using a Beckman System Gold HPLC (Beckman Coulter, USA). Liposomes containing Quer were disrupted with methanol, leading to the solubilization of Quer. The solution was then filtered through a PTFE membrane with 0.2 μm pore diameter before quantification at 360 nm. The mobile phase consisted of methanol/water acidified with 0.1 % (v/v) trifluoroacetic acid (70:30, v/v).

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

$$\eta_{Quer}(\%) = \frac{amount\ of\ Quer\ after\ SASD\ (\mu g)}{amount\ of\ Quer\ before\ SASD\ (\mu g)} \times 100 \quad (3)$$

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

$$\eta_{lipid}(\%) = \frac{amount\ of\ lipid\ after\ SASD\ (\mu mol)}{amount\ of\ lipid\ before\ SASD\ (\mu mol)} \times 100 \quad (4)$$

After SASD processing, upon liposomes resuspension the *IE* of the Quer is given by the following equation:

$$I.E._{\cdot ress}(\%) = \frac{\left(\frac{\mu g_{Quer}}{\mu mol_{Lip}}\right)_{ress}}{\left(\frac{\mu g_{Quer}}{\mu mol_{Lip}}\right)_f} \times 100 \quad (5)$$

where $\left(\frac{\mu g_{Quer}}{\mu mol_{Lip}}\right)_f$ is the Quer-to-lipid ratio before SASD and $\left(\frac{\mu g_{Quer}}{\mu mol_{Lip}}\right)_{ress}$ is the final ratio after SASD and liposomes resuspension.

2.2.5. Characterizations of liposomal dry powder formulations

2.2.5.1. In vitro aerosolization study. The aerodynamic properties of the Pos_Lip-DPFs and Quer_Pos_Lip-DPFs were determined using an eight-stage Andersen Cascade Impactor – ACI - (Copley Scientific, UK). Soon thereafter, 30 mg of Quer_Pos_Lip-DPFs and Pos_Lip-DPFs were loaded into three hydroxypropylmethylcellulose capsules nº3 (Aerovaus), respectively. 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, Spain). The DPI punctured the capsule before the inhalation, and a high-capacity pump was turned on to simulate an intake of breath: an airflow rate of 60 L/min lasting 4 s, according to the European pharmacopoeia [28]. 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), and geometric standard deviation (GSD). The MMAD was determined as the particle diameter corresponding to 50 % of the cumulative mass distribution, excluding those deposited in the throat. On the other hand, FPF refers to

the fraction of delivered particles with a diameter below 5 μm, determined by interpolating the percentage of particles smaller 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.

2.2.5.2. Scanning electron microscopy/ energy-dispersive X-ray spectrometry (SEM-EDS). The shape and morphology of Lip-DPFs particles were determined by scanning electron microscopy (SEM). The samples were coupled to adhesive carbon tapes, and the excess powder was removed by a jet of compressed air. Then a Hitachi S2400 with Bruker light elements *energy-dispersive X-ray spectrometry* (EDS) detector (Japan) analysed the samples with an accelerating voltage set to 15 kV and at magnifications of 5 k and 10 k.

2.2.5.3. Residual moisture content. Residual ethanol content was evaluated by high-performance liquid chromatography (HPLC) Dionex ICS3000 (USA) with Pulsed Amperometric Detection (PAD). Briefly, Lip-DPFs with and without Quer were dissolved in Milli-Q water at 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 (USA). The analysis was conducted at a flow rate of 1 mL/min with NaOH (18 mM).

2.2.5.4. In vitro cytotoxic studies. L929 mouse areolar fibroblast cells (NCTC clone 929, ATCC) were used for the cytotoxicity studies. This fibroblast-like cell line, derived from subcutaneous areolar connective tissue, is widely employed in cytotoxicity assays as a standard model for assessing the biocompatibility of materials and active molecules (ISO 10993–5). Its relevance stems from the abundance of areolar connective tissue in vertebrates, where it provides a protective framework that stabilizes major organs and structures. L929 cells were seeded at a density of 10^5cells/mL (ISO 10993–5) into 96-well plates and incubated in culture medium (α-MEM supplemented with 10 % (v/v) FBS, 100 IU/mL penicillin, 100 μg/mL streptomycin and 2.5 μg/mL amphotericin B), for 24 h at 37 °C, in a humidified atmosphere (5 % CO_2/air). After incubation, adhered cells were treated with the different formulations, for 6 and 24 hours. Cell viability was assessed by the MTT assay. Cultures performed in the absence of the materials were used as controls. All the experiments were performed at least in triplicate.

2.2.6. Statistical analysis

All results are expressed as mean ± standard deviation (S.D.). To analyse the data from *in vitro* cytotoxic studies, analysis of variance (ANOVA) followed by Bonferroni post-test (GraphPadPrism, GraphPad software100 Inc., USA) was used. The level of significance was set at the probabilities of $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

3. Results and discussion

3.1. Optimization of liposomes and quercetin-loaded liposomes

The biggest challenge faced in drying drug-loaded liposomal formulations is to keep their size and the drug (Quer) incorporated. To address this challenge, the composition of the lipid bilayer, including surface charge and molar ratios of lipid/Quer was varied (as summarized in Table 1). Neutral, negative, and positive-surface charged Quer-loaded liposomes (*Quer_Neut_Lip*, *Quer_Neg_Lip*, and *Quer_Pos_Lip*, respectively), with molar ratios of lipid/Quer 10:0.5 and 10:1 were used.

Table 1 shows that no major differences in size between *Pos_Lip* and *Neut_Lip*. However, a slight increase in size was observed when Quer was added to Pos_Lip. Eid *et al.* [29] observed, through atomic force microscopy and molecular dynamics measurements on liposomes composed of PC, Chol and Quer, that an increase in Quer concentration led to greater distances between lipid molecules, resulting in larger liposomes. In this case, the size increase is only evident when both Quer and SA are present in the lipid bilayer. Although the presence of Quer and SA individually does not impact liposomes size, combined presence leads to an increase in size. Further atomic characterization and computational modelling are necessary to elucidate this synergistic effect. Regarding the surface charge, the ζ-potential increased for *Pos_Lip* and *Quer_Pos_Lip*, indicating successful SA distribution in the liposome bilayer. Finally, 93–99 % of Quer was incorporated into the liposome bilayer.

Table 1
Physicochemical characterization and incorporation efficiency (*I.E.*) of *Quer_Neut_Lip*, *Quer_Neg_Lip*, and *Quer_Pos_Lip*, before SASD.

Formulation	Lipid/Quer molar ratio	Size [nm]	PdI	ζ-potential [mV]	(Quer/Lip)$_f$ [μg/μmol]	*I.E.* [%]
Quer_Neg_Lip	10:0.5	137 ± 14	0.089 ± 0.048	- 24 ± 2	12 ± 5	92 ± 3
	10:1	147 ± 1	0.114 ± 0.017	- 28 ± 1	35 ± 6	89 ± 1
Neut_Lip	-	136 ± 1	0.098 ± 0.016	- 1 ± 1	-	-
Quer_Neut_Lip	10:0.5	150 ± 5	0.113 ± 0.029	- 1 ± 1	19 ± 4	99 ± 15
	10:1*	146 ± 8	0.150 ± 0.002	- 1 ± 1	36 ± 2	84 ± 12
Pos_Lip	-	137 ± 5	0.080 ± 0.006	11 ± 1	-	-
Quer_Pos_Lip	10:0.5	195 ± 2	0.093 ± 0.016	14 ± 1	15 ± 3	99 ± 5
	10:1*	171 ± 18	0.123 ± 0.007	13 ± 1	34 ± 6	93 ± 5

The results are expressed as mean ± S.D (n = 3).
Quer_Neg_Lip - DMPC: DMPG:Quer in a molar ratio of 7:3:1; *Quer_Neut_Lip* -E-PC: Chol:Quer in a molar ratio of 9:1:1;
Quer_Pos_Lip - E-PC:Chol:SA:Quer in a molar ratio of 8:1:1:1; PdI: Polydispersity index
* n = 6 independent batches

As mentioned earlier, maintaining the structural integrity of liposomes during and after SASD processing, as well as retaining the incorporated molecule (even after dissolution of the powder) can pose challenges. Costa *et al.* [21,26] previously optimized SASD conditions to convert liposomes loaded with biopharmaceutical or a hydrophilic molecule of low molecular weight into solid dosages forms. The authors observed that at specific process parameters and percentage of leucine and ethanol, liposomes could retain their integrity, with the encapsulated molecules remaining into the internal aqueous core [21]. For biopharmaceuticals, it was observed that even under moderate pressures and high temperatures, the enzymatic activity of the enzyme was preserved [26]. Based on these observations, in the current study, Neut_Lip, *Pos_Lip*, *Quer_Neut_Lip* and *Quer_Pos_Lip* were processed in SASD (Table 2).

The process yield ranged between 48 % and 65 % of the recovered powder, with liposomes successfully encapsulated in trehalose and leucine. EDS analysis revealed the presence of 49.4 ± 0.1 % of carbon atoms, 47 ± 1 % oxygen atoms, and a residual presence of nitrogen atoms (3.2 ± 0.9 %), probably from liposomes present on the sugar surface. Following SASD processing, for resuspendability test and liposomes characterization, all the Lip_DPFs and respective controls were resuspended in deionized water to maintain their osmolarity. Ultracentrifugation was then utilized to remove the excipients. After liposomes reconstitution, it was observed (Table 3) that *Quer_Neg_Lip* exhibited a lower *IE* (13–14 %) compared to *Quer_Neut_Lip* or *Quer_Pos_Lip*. Furthermore, liposomes with molar ratios of 10:1 presented

Table 2
SASD process parameters used to convert Quer_Neg_Lip, Quer_Neut_Lip, and Quer_Pos_Lip into Quer_Neg_Lip-DPFs, Quer_Neut_Lip-DPFs, and Quer_Pos_Lip-DPFs.

		p_{SM} [bar]	T_{SM} [℃]	T_{in} [℃]	T_{out} [℃]	Yield Process [%]	Water content [%]
Quer_Neg_Lip-DPFs	10:0.5	121 ± 1	91 ± 4	99 ± 7	66 ± 5	58	6
	10:1	120 ± 1	84 ± 10	101 ± 8	66 ± 5	65	6
Neut_Lip-DPFs	-	119 ± 1	96 ± 2	98 ± 2	57 ± 3	64 ± 2	-
Quer_Neut_Lip-DPFs	10:0.5	121 ± 1	92 ± 3	93 ± 2	63 ± 0.2	66	6
	10:1*	121 ± 2	83 ± 7	98 ± 4	66 ± 5	48 ± 4	7 ± 1
Pos_Lip-DPFs	-	118 ± 1	92 ± 1	97 ± 4	56 ± 3	60 ± 6	-
Quer_Pos_Lip-DPFs	10:0.5	120 ± 1	83 ± 7	102 ± 7	68 ± 1	65	5
	10:1*	121 ± 1	80 ± 5	101 ± 8	66 ± 1	56 ± 5	6 ± 1

Lip-DPFs: Liposomal dry powder formulations
$T_{air,in}$: Temperature of heated compressed air; $T_{CO2:}$ Temperature of heated CO_2; $T_{air,out:}$ Outlet air temperature; T_{SM}: Temperature at the static-mixer; p_{SM}: Pressure at the static-mixer.
Quer_Neg_Lip - DMPC: DMPG: Quer in a molar ratio of 7:3:1; *Quer_Neut_Lip* - E-PC:Chol:Quer in a molar ratio of 9:1:1;
Quer_Pos_Lip - E-PC:Chol:SA:Quer in a molar ratio of 8:1:1:1
* n = 6 independent batches

Table 3
Physicochemical characterization and incorporation efficiency (*I.E.*$_{ress}$) of reconstituted *Quer_Neut_Lip*, *Quer_Neg_Lip*, and *Quer_Pos_Lip*, after SASD.

	Molar ratio	Size [nm]	PdI	ζ- potential [mV]	Lipid [μmol/mL]	$(Quer/Lip)_f$ [μg/μmol]	*I.E.*$_{ress}$ [%]
Quer_Neg_Lip	10:0.5	117	0.139	−7.2	71	0.9	14
	10:1	150	0.240	−12.2	65	2.7	13
Neut_Lip	-	121 ± 2	0.229 ± 0.009	- 0.8 ± 0.3	55 ± 10	-	-
Quer_Neut_Lip	10:0.5	146	0.240	−2.7	45	13	37
	10:1	143 ± 3	0.378 ± 0.008	0.6 ± 0.4	67 ± 1	44 ± 1	66 ± 3
Pos_Lip	-	260 ± 2	0.412 ± 0.006	10.6 ± 0.2	82 ± 7	-	-
Quer_Pos_Lip	10:0.5	240	0.376	4.1	60	9	28
	10:1	252 ± 37	0.420 ± 0.036	10 ± 1	84 ± 13	37 ± 7	62 ± 11

Neg_Lip - DMPC: DMPG: Quer in a molar ratio of 7:3;
Quer_Neg_Lip - DMPC: DMPG: Quer in a molar ratio of 7:3:1;
Neut_Lip - E-PC:Chol in a molar ratio of 9:1;
Quer_Neut_Lip - E-PC:Chol:Quer in a molar ratio of 9:1:1;
Pos_Lip - E-PC:Chol:SA in a molar ratio of 8:1:1; *Quer_Pos_Lip* - E-PC:Chol:SA:Quer in a molar ratio of 8:1:1:1; PdI: Polydispersity index; $(Quer/Lip)_f$: Final quercetin to lipid ratio considering the final lipid concentration value
*n = 6 independent batches

higher IE than those with molar ratios of 10:0.5 for *Quer_Pos_Lip*. Besides, *Neut_Lip* showed a smaller size than before supercritical processing, consistent with findings from previous studies [21,26].

According to the water replacement theory, during drying, water molecules are replaced by carbohydrates, which establish hydrogen bonding with the phospholipids' polar head [30]. Leucine also plays an important role in maintaining the liposomes' size, as electrostatic interaction between the carboxyl group of the Leu and the E-PC amine group contribute to liposomal stabilization [31]. Moreover, studies of Pal *et al.* [32] demonstrated that an increase in ethanol concentration contributes to liposome shrinking, due to a reduction in the bending modulus of lipid membranes. In fact, after SASD, *Neut_Lip* decreases their size from 136 ± 1–121 ± 2 nm, while its PdI increased from 0.098 ± 0.016–0.229 ± 0.009, suggesting an increase in the size heterogeneity due to liposome rearrangement during the processing. Indeed, we hypothesize that in the static mixer, when $scCO_2$ is rapidly mixed with the water/ethanol solution containing liposomes, its diffusion into the bilayer increases. This may result in a higher polydispersity index and liposome rearrangement.

Regarding Quer_Neut_Lip, an increase in size was observed. This might be related to the presence of Quer in the lipidic bilayer, as its addition in lipidic bilayers contributes to larger distances between the lipid molecules [29]. Studies from Movileanu *et al.* [33] demonstrated that Quer molecules penetrate the lipid bilayer by intercalating between the flexible acyl chains of the phospholipids. Regarding the *Pos_Lip*, it was observed that the insertion of SA in the lipidic bilayer led to a higher increase in size and PdI compared to *Neut_Lip*, after SASD processing. Toopkanloo *et al.* [34] suggested that highly lipophilic agents induced structural changes in liposome membrane and vesicle stability, changing fluidity and packing density of the polar-nonpolar interface of the phospholipid head group area at the liposome surface, similar to the effect of cholesterol. Therefore, under thermal and shear stress, an increase in size is expected. Regarding Quer's *IE*, similar values were obtained for both formulations. The decrease in *IE* may be attributed to thermal and air-interface-related stresses. The shear rate imposed on liposomes resulted in the unfolding and exposure of the hydrophobic bilayer [35]. It is important to note that HPLC results demonstrated no modification of quercetin's structure after SASD processing (Figure S1-Supplementary material).

Thermodynamic studies of the solubility of Quer in a CO_2 + ethanol system by Chafer and co-workers [36] have shown that the solvating power of CO_2 increases with pressure, leading to more solute is transferred to the supercritical phase. To obtain a better understanding, studies were conducted with Quer/trehalose/leucine system (without liposomes). It was observed that only 25 ± 3 % of Quer remained in the final powders.

The ζ- potential of resuspended *Quer_Pos_Lip* decreased to 10 ± 1 mV. A positive surface contributes to a better interaction with the negatively charged cell surface, potentially promoting either endocytosis or Quer diffusion through the liposome's bilayer to cells. To assess whether an increase in the percentage of leucine and ethanol would benefit the *IE*, additional studies were performed. The leucine percentage was increased to 15 % and ethanol to 30 %. As expected, better results were obtained in terms of size (225 ± 27 nm), while the PdI value remained similar (0.426 ± 0.029), compared to *Quer_Pos_Lip*. However, the *IE* decreased to 32 ± 8 %, indicating that even higher

Table 4
Aerodynamic and chemical characterization of Pos_Lip-DPFs and Quer_Pos_Lip-DPFs.

	MMAD [μm]	GSD	FPF [%]	*Residual ethanol [ppm]*
Pos_Lip-DPFs	1.7 ± 0.1	2.3 ± 0.1	59 ± 7	< 5000
Quer_Pos_Lip-DPFs	1.7 ± 0.1	2.2 ± 0.1	63 ± 4	< 5000

The results are expressed as mean ± S.D (n = 3).

concentrations of leucine and ethanol cannot minimize the Quer loss from liposomes. Due to the similarity of the results obtained for the neutral and positively surface charge liposomes, in terms of liposomal properties and with the objective of maximizing liposomes (positive surface charge) interaction with cells, further experiments were carried out with *Pos_Lip* and *Quer_Pos_Lip*.

3.2. Aerodynamic properties of Pos_Lip and Quer_Pos_Lip

Following this, aerodynamic, and morphological characterization of empty and quercetin- liposomal dry powder formulations were then performed (Figure S2 - Supplementary material). Table 4 shows the values obtained for Quer_Pos_Lip-DPFs and Pos_Lip-DPFs.

Quer_Pos_Lip-DPFs showed an MMAD of 1.7 μm and a fine particle fraction (FPF) of 63 %, facilitating their deposition in the alveolar airspaces within the respiratory region. The same was observed for *Pos_Lip-DPFs*, as corroborated by SEM images (Fig. 1).

According to the aerodynamic studies, 57 ± 2 mg of powder reaches the respiratory tract, resulting in 5.2 ± 0.4 μg of delivered Quer from liposomes. As Quer is classified as a class IV drug by the Biopharmaceutics Classification System, higher doses in its free form are typically required for efficient treatment. Work from Mamani-Matsuda *et al.* [37] demonstrated that five oral administrations of Quer at a dose of 160 mg/kg to arthritic rats resulted in an anti-arthritic effect compared to untreated control. Similar results were observed for those treated with five cycles of 60 mg/kg administered via the intra-peritoneal route. However, studies from Priprem and co-workers [38] showed a significant reduction in the required dose of Quer for anti-inflammatory purposes when incorporated into liposomes and administered intranasally. Their study compared the oral intake of 300 mg/kg/day of free Quer in rats with the intranasal administration of 20 μg/day of Quer-loaded liposomes. The results showed that a lower dose and a faster rate of anxiolytic effects were observed for liposomes administrated via intranasal route compared to oral administration.

3.3. In vitro cytotoxicity assays

To assess the potential toxicity of formulations with and without Quer, *in vitro* cell viability studies were conducted using L929 (areolar fibroblasts) with incubation times of 6 and 24 h. Previous studies have indicated that the equivalent concentration of 10 μM of Quer does not exhibit toxicity in cells [13]. Based on this and the incorporation efficiency, the *in vitro* studies were conducted, ranging the lipid concentration from 3 μM to 126 μM (corresponding to 0.1 μM to 10 μM of Quer).

Pos_Lip and Quer_*Pos_Lip*, reconstituted *Pos_Lip* and *Quer_Pos_Lip* from powder (rehy. *Pos_Lip* and rehy. *Quer_Pos_Lip*, respectively), and *Pos_Lip-DPFs* and *Quer_Pos_Lip-DPFs* were incubated on cells for 6 h and 24 h. At both time-points (Fig. 2), cell viability remained above 85 % for all formulations, with cell behaviour identical to the control group. A slight but significant decrease of cell viability, measured by MTT cleavage at 6 hours, was observed for unloaded liposomes at a concentration of 126 μM (*p-value* < 0.0014) and *Quer_Pos_Lip* at the same concentration (*p-value* < 0.0001). This effect was reversed after 24 h. The rapid colorimetric assay relies on the cleavage of the tetrazolium ring of MTT (3-(4,5-dimethylthazolk-2-yl)-2,5-diphenyl tetrazolium bromide) by dehydrogenases in active mitochondria of living cells to estimate viable cell number. Consequently, it can be hypothesised that the decrease in cell viability observed after 6 hours is not due to a toxic effect but rather a delayed reaction of MTT with mitochondria, essentially due to the high internalization of liposomes by the cells. This hypothesis is sustained by the cell viability observed at 24 hours. Nevertheless, cell viability remained above 85 % for all time points and the formulations.

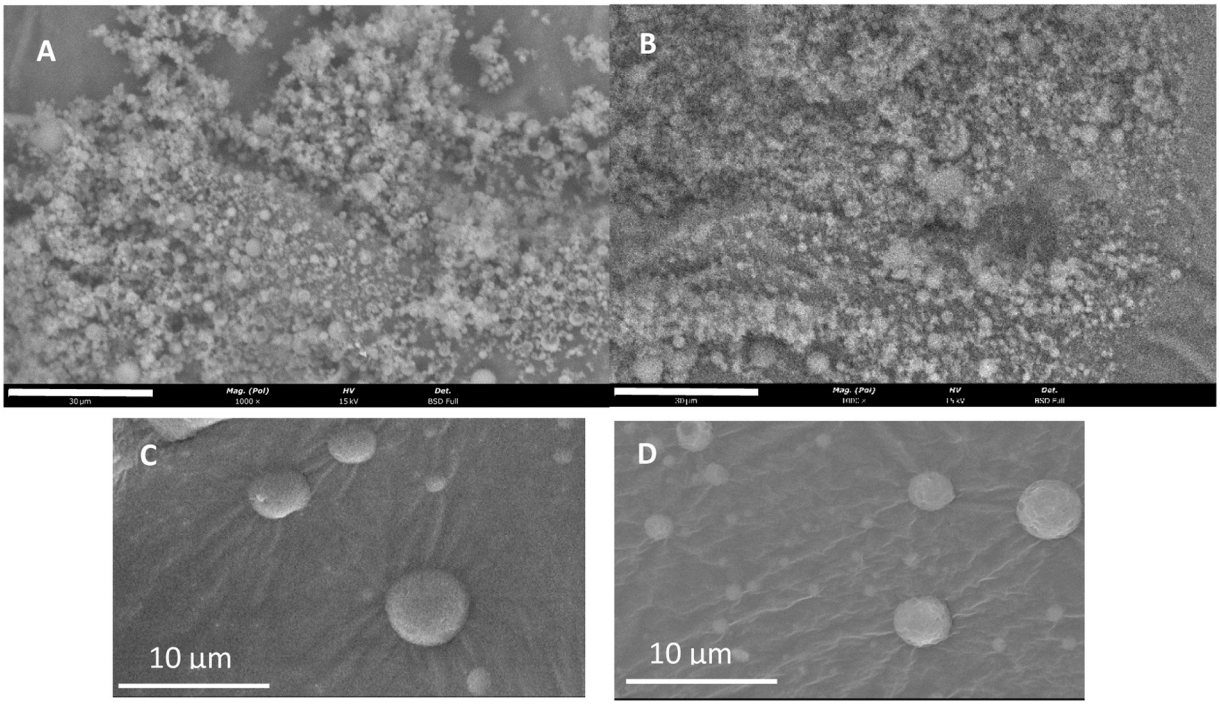

Fig. 1. SEM images of (A),(B) and (C) Quer_Pos_Lip-DPFs and (D) Pos_Lip-DPFs ((A) and (B) scale bars: 30 μm; (C) and (D) scale bars: 10 μm; magnitude: 3000 x; high-voltage: 15 kV).

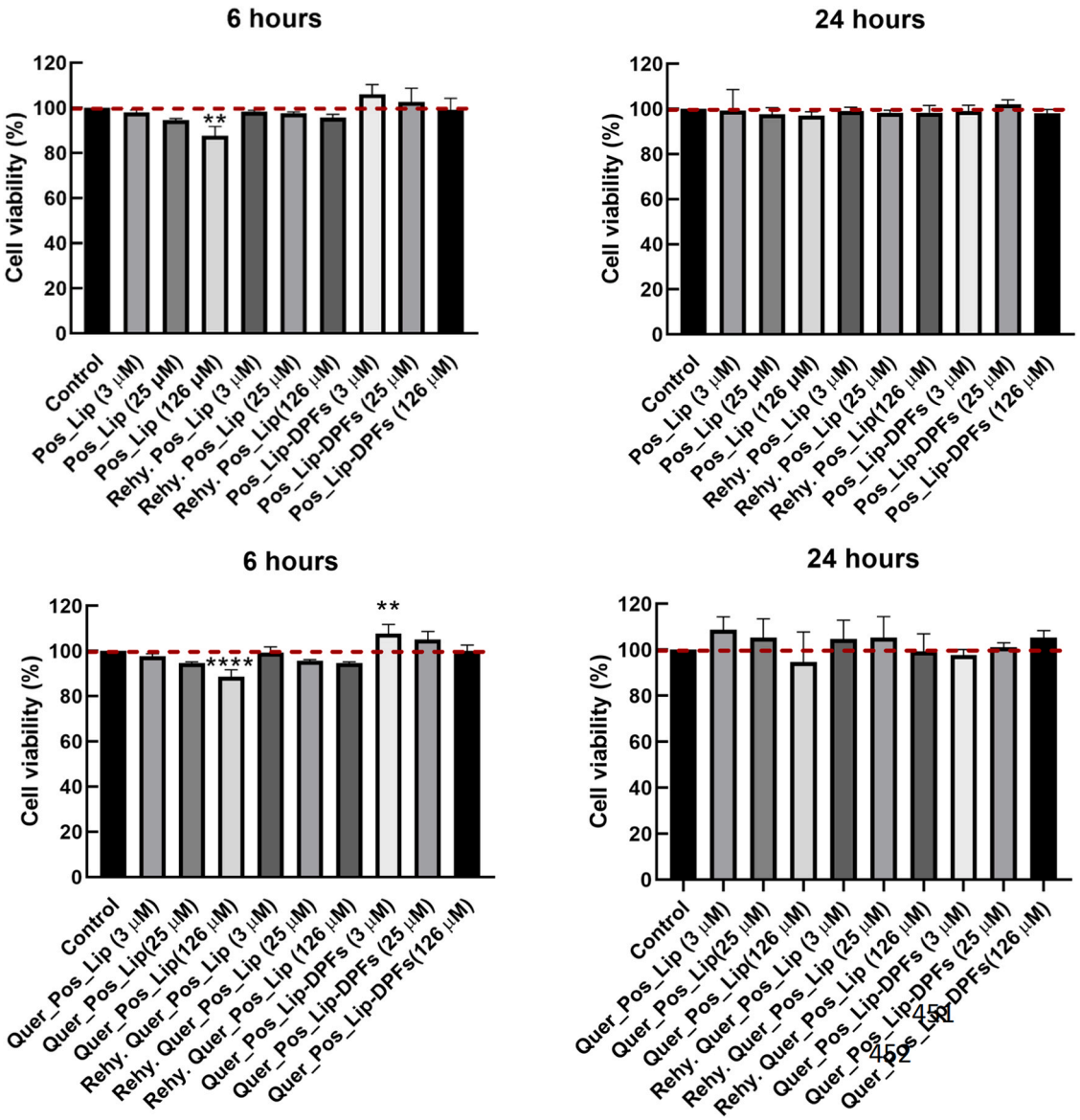

Fig. 2. L929 cell lines viability when exposed to different concentrations of Pos_Lip and Quer_Pos_Lip, reconstituted Pos_Lip and Quer_Pos_Lip from powder (rehy. Pos_Lip and rehy. Quer_Pos_Lip, respectively), and Pos_Lip-DPFs and Quer_Pos_Lip-DPFs, after 6 h and 24 h of incubation at 37 °C. All the data were compared to the negative control. The level of significance was set at the probabilities of $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$. The results are expressed as mean ± S.D. (n = 3).

4. Conclusions

This work focused on optimizing Quer-loaded liposomal dry powder formulations by utilising supercritical CO_2-assisted spray-drying to convert liposomal suspensions loaded with hydrophobic compounds into liposomal dry powder formulations. The primary challenge of this study was to preserve the structural integrity of liposomes and achieve an incorporation efficiency of over 80 %, after the drying process. To achieve this, various compositions of liposome membranes and molar ratios of lipid/Quer were explored to enhance the incorporation efficiency. Quer_Pos_Lip formulation was selected for further studies considering the improved incorporation efficiency. During the SASD process, thermal and air-interface-related stresses may result in the unfolding and exposure of the hydrophobic bilayer. Additionally, it is hypothesized that CO_2 may have the ability to drag Quer during the process. Nonetheless, an *IE* of 57 ± 10 % ($n = 3$) was achieved. The incorporation of Quer, along with the aerodynamic performance of the dry powders, demonstrates that Quer_Pos_Lip-DPFs exhibit aerodynamic properties necessary for effective pulmonary liposomal drug delivery. *In vitro* studies revealed no toxicity in fibroblasts for both reconstituted Quer_Pos_Lip and Quer_Pos_Lip-DPFs, after 6 h and 24 h. Despite not achieving an *IE* above 80 %, this study marks a beginning of a new approach to enhance the solubility and bioavailability of Quer for inhalation purposes and the treatment of lung-associated diseases by using green, scalable and sustainable drying technique.

CRediT authorship contribution statement

Gomes Pedro Sousa: Writing – review & editing, Resources, Methodology. **Casimiro Teresa:** Writing – review & editing, Methodology, Investigation. **Grenho Liliana:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Costa Clarinda:** Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Aguiar Ricardo Ana:** Visualization, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization. Writing – review & editing. **Corvo M. Luisa:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Methodology,

Funding acquisition, Formal analysis, Conceptualization. **Fernandes Maria Helena:** Writing – review & editing, Validation, Resources, Methodology.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Clarinda Costa reports financial support was provided by Foundation for Science and Technology. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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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.2025.106591.

Data availability

Data will be made available on request.

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