# Liposomes Size Engineering by Combination of Ethanol Injection and Supercritical Processing

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**ABSTRACT:** Supercritical fluid extraction using a high-pressure packed tower is proposed not only to remove the ethanol residue from liposome suspensions but also to affect their size and distribution leading the production of nanosomes. Different operating pressures, temperatures, and gas to liquid ratios were explored and ethanol was successfully extracted up to a value of 400 ppm; liposome size and distribution were also reduced by the supercritical processing preserving their integrity, as confirmed by *Z*-potential data and Trasmission Electron Microscopy observations. Operating at 120 bar and 38°C, nanosomes with a mean diameter of about 180 ± 40 nm and good storage stability were obtained. The supercritical processing did not interfere on drug encapsulation, and no loss of entrapped drug was observed when the water-soluble fluorescein was loaded as a model compound. Fluorescein encapsulation efficiency was 30% if pure water was used during the supercritical extraction as processing fluid; whereas an encapsulation efficiency of 90% was obtained if the liposome suspension was processed in water/fluorescein solution. The described technology is easy to scale up to an industrial production and merge in one step the solvent extraction, liposome size engineering, and an excellent drug encapsulation in a single operation unit.
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## INTRODUCTION

Over the last decades, different drug delivery systems have been intensively investigated. One of them is liposome, a colloidal association of amphiphilic lipids that organize themselves spontaneously in bilayer vesicles, as a result of unfavorable interactions between phospholipids and water.[1–3] Liposomes showed to be effective carriers for the delivery of various kinds of agents into cells; indeed, lipid vesicles present lipophilic and hydrophilic portions that can entrap substances with different lipophilicities in the phospholipid bilayer, in the aqueous compartment or either at the bilayer interface.[4–6]

Several techniques are reported in the literature for the preparation of liposome suspensions. The first one involves the dissolution of phospholipids into an organic solvent and, then, dispersion of the dried lipids into water (hydration method); hydration reduces the energy of the system and causes the increase of its specific surface area, leading to the maximum exposition of the polar heads to water top, so liposomes are formed.[7] Using sonication or high-speed mixing, it is then possible to vary the distance between the lipid lamellae and to influence the size of multilamellae vesicles or the formation of unilamellae ones.[8,9] Hydration method is relatively easy, but produces liposomes with large polydispersity or bimodal size distribution and low entrapped volume; moreover, it has a limited use because of its low encapsulation ability and difficult process scale up. Reverse-phase evaporation method has also been described starting from double water–oil–water emulsions evaporation; in this case, the drug is loaded into the water internal phase and the liposome components are solubilized into the oily phase; the lipid membrane is, then, formed by solvent evaporation of the organic solvent of the oily phase. The liposome size is defined by the droplet size and a high entrapping efficiency is also reported.[10–12] The main drawback of this method is the use of organic solvents such as chlorophorm, isopropylether, and methylene chloride that have to be removed from the bulk of liposomes suspension. The rapid injection of lipids dissolved in ethanol into a water or water/surfactant solution is reported as a robust technique for liposomes production and it is conventionally named ethanol injection; it can produce small unilamellar vesicles by using different strategies for the organic and water solution mixing. The technique was developed by Batzri and Korn[13] and optimized by several authors up to the pilot scale, because it was reported as a simple, rapid, and easy to scale up method. The liposomes size, encapsulation efficiency lamellarity, and stability are well controlled and reproducible.[14,15]

Although various techniques have been developed for the bench-scale liposome formation, the broad application of liposomes in drug delivery is still hindered by scale-up issues. Liposome formation processes should be validated according to good manufacturing practice protocols prior to commercialization. Particularly, all the commercial techniques proposed and scaled-up showed a consistent difficulty to remove solvents from the liposomes membrane and from the bulk of the suspension, leading to a serious drawback for process industrialization. Although these organic solvents help to achieve a molecular mixture of lipids and, therefore, a uniform distribution of lipids in the lipid bilayers, their residual amount in the final preparation represent a health hazard to the end users. Moreover, solvent evaporation technology, conventionally used for

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solvents removal, tends to concentrate the lipids, as well as the contaminants. Ethanol removal is also difficult and may lead to physical destabilization of liposomes by interfering with the cooperative hydrophobic interactions among the phospholipid methylene groups that hold the structure together.[9,16,17]

Supercritical fluid technologies have been recently tested in several extraction applications for new process/material production.[18–21] The utilization of supercritical fluids for solvent extraction from emulsions and/or suspension has also been recently proposed as a new technique for the production of biopolymer microspheres. The extraction technology can overcome several disadvantages of the conventional ones, such as high processing temperatures and long extraction times. Particularly, above the critical point, small changes in temperature or pressure can produce large changes in the density/solvation ability of supercritical fluids; this property can be fruitfully exploited for the extraction of organic solvents. In addition, the lower viscosity and the higher diffusivity of a supercritical fluid with respect to the liquid solvent improve mass transfer, which is often the limiting factor for the solvent elimination from emulsions or suspensions.[22–24] Among all the possible supercritical fluids, carbon dioxide (SC-$CO_2$) is largely used. Recent studies confirmed that the SC-$CO_2$ is an excellent solvent for oily phase upon contact with the aqueous phase of the emulsion, leading to rapid diffusion of solvent from the emulsion droplets; the process is also faster than the conventional solvent evaporation of emulsion, resulting in the prevention of any particles coalescence or aggregation.[25]

Some authors also reported methods for the preparation of liposomes using carbon dioxide–ethanol supercritical mixtures and sometimes vesicles with smaller mean sizes were produced with respect to the conventional technologies. As an example, unilamellar vesicles with a diameter of about 200 nm were reported by a rapid expansion of a homogeneous phospholipid–ethanol–$CO_2$ mixture and simultaneous mixing with an aqueous solution by passing the resultant gas–water mixture through a specially designed mixing column;[26] the method can be regarded as a rapid expansion of supercritical solution technology. Phospholipid vesicles with diameters from 200 nm to 20 µm were also reported by several authors[27–29] by using the supercritical technology and ethanol–carbon dioxide mixtures. Recently, Espirito Santo et al.[30] also reported a continuous supercritical fluid process, named supercritical assisted liposome formation to prepare nanosomes of controlled size, using an expanded liquid mixture formed by phospholipids–ethanol–carbon dioxide. However, in all the described techniques, the ethanol residue in the produced suspensions is still an issue to be solved.

The production of liposomes utilizing only SC-$CO_2$ as an alternative to organic solvents was also proposed for the production of liposomes of various phospholipids by depressurizing the supercritical phase into water[28]; using pressures over 200 bar, spherical vesicles with good uniformity were obtained, in the absence of any organic solvent, including ethanol.[31] Other authors also reported a similar discontinuous method with the formation of an oil-in-water emulsion then mixed with SC-$CO_2$ by a static mixer; the biphasic mixture is then depressurized down to atmospheric pressure using a capillary nozzle producing a very intense atomization of the emulsion, which facilitates the formation of liposomes.[32] The authors reported fairly good encapsulation efficiency on vesicles size control. Liposomes with a mean diameter of 0.4–1.3 µm and an efficiency of incorporation of essential oil of up to 66% were described.

The purpose of this work is the use of supercritical carbon dioxide to extract ethanol from liposome suspensions previously fabricated by ethanol injection, by using a high-pressure packed column avoiding at the same time vesicles degradation or loss of the entrapped drug. The vesicles size reduction after supercritical processing will also be explored. Different liposomes suspensions were prepared by ethanol injection method at different phospholipid concentrations; they were, then, processed to evaluate the effect of supercritical processing parameters such as, various pressures, temperatures, and liquid to gas ratio, on liposomes stability and size distribution. Solvent residues were always measured at the end of each supercritical processing to verify the process efficiency. Liposome size and distribution and their integrity at the end of the extraction process were also monitored by laser granulometer, Z-potential measurement of the suspensions, and by Trasmission Electron Microscopy observation of the vesicles before and after supercritical processing. Water-soluble fluorescein was also added as a model compound into the vesicles to ascertain whether the proposed technology interferes on drug encapsulation.

## MATERIALS AND METHODS

### Materials

Soybean phosphatidylcholine (Soy PC) was provided by Lipoid (Ludwigshafen, Germany). Fluorescein sodium salt was purchased from Sigma–Aldrich (Milan, Italy); its solubility in water was 500 g/L. Distilled water was used throughout the formulations. $CO_2$ (99.9%) was purchased from SON (Naples, Italy). All the other reagents and organic solvents were, at least, of analytical grade and used such as without further purification.

### Liposome Production

Liposomes were prepared by the ethanol injection method adapted from Justo and Moraes.[33] Briefly, Soy PC was dissolved in heated ethanol (40°C) to improve the miscibility of the phospholipid into the organic solvent. An ethanol solution of Soy PC was continuously added dropwise into water through a stainless steel needle. Ethanol and water were used in the ratio 5/95. Liposomes suspensions were then sonicated using the Digital Sonifier Branson (mod. 450, ½″ diameter microtip, 20 kHz) at 60% of the power for 2 min. Lipid concentration was varied from 5 to 15 mM. Fluorescein encapsulation into liposome was obtained by using a water solution of 5 mg/mL of fluorescein in water.

### Continuous Supercritical Fluid Extraction

The apparatus consists of a stainless steel packed column with an internal diameter of 13 mm, in which carbon dioxide is fed from the bottom of the column using a high-pressure diaphragm pump, and the liposome suspension is delivered from the top of the column using a high-pressure piston pump. The column is formed by three AISI 316 stainless steel cylindrical sections of 30 cm height, connected by four cross-unions and is filled with stainless steel packing elements of 4 mm nominal size with 1889 $m^{-1}$ specific surface and 0.94 of voidage (0.16 inch Pro-Pak; Scientific Development Company, State College, Pennsylvania). The apparatus is thermally insulated by ceramic cloths and its temperature is controlled by six controllers

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