LIPOSOME PREPARATION USING A NEW SUPERCRITICAL FLUID ASSISTED CONTINUOUS PROCESS

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ABSTRACT

Liposomes are formed by phospholipids that spontaneously generate vesicles as a consequence of their interactions with water. In this work, a new continuous supercritical fluid process, named Supercritical Assisted Liposome formation (SuperLip), is proposed to prepare liposomes of controlled submicrometric size. Water droplets are produced by atomization inside a high pressure vessel, filled with an expanded liquid mixture formed by phospholipids/ethanol/Carbon dioxide (CO_2). These droplets are rapidly surrounded by a lipid layer and liposomes are formed when they fall in the water pool located at the bottom of the vessel. Liposomes with controlled dimensions and high encapsulation efficiency, containing water soluble drugs, can be generated. Experiments have been performed varying process operating parameters like pressure and temperature, producing liposomes of soybean phosphatidylcholine (PC) of different size and distribution ranging between 130 ± 62 nm and 294 ± 144 nm. The results demonstrated that atomized liquid droplets are transformed efficiently into liposomes. Also phosphatidylglycerol (PG) had been used coupled with PC to produce liposomes. PC/PG larger liposomes have been produced in this case ranging between 442 ± 110 nm and 209 ± 72 nm. Drug encapsulation feasibility tests were also performed using bovine serum albumin (BSA), used as a model protein; high encapsulation efficiencies (85 ÷90%) were obtained, confirming that the active compound contained in the water phase was efficiently entrapped in the formed vesicles.

INTRODUCTION

Liposomes are small lipid vesicles containing water suspended in a water based medium. They can be also defined as water in water (w/w) emulsions and can contain hydrophilic active principles dissolved in the water phase or hydrophobic compounds in the space between layers, when multilayer vesicles are formed [1].
Conventional technologies, used to produce liposomes, consist of several preparation steps yielding low batch-to-batch uniformity; in several cases, low encapsulation efficiencies are also reported [2, 3]. In the field of particle formation and production of delivery vesicles, supercritical fluid technologies can overcome several limitations of conventional processes. Recently, some techniques based on the use of supercritical CO_2 ($scCO_2$) have been proposed also for liposomes preparation [4-9]; they tried to take the advantage of the enhanced mass transfer of supercritical fluids [10] and can be roughly divided in two categories: (a) *two steps processes* in which the dried lipid particles need to be, then, rehydrated [8, 11-15]; (b) *one step processes* in which a liposome water suspension is directly obtained at the end of the process [1].
These kind of processes has some drawbacks related to the control of particles dimension and distribution and only batch or semi-continuous layout has been proposed. Furthermore, they show a common limitation from the point of view of the possibility of encapsulation (ranging around 10÷20%) because both in the one step processes and in the two step processes only a small part of the solution used to hydrate liposomes, that contains the drug to be entrapped, is effectively included in the lipidic bilayer.
In this work a new one step continuous supercritical CO_2 based process is proposed, named Supercritical Assisted Liposome formation (SuperLip). Differently from the previously proposed processes, we tried to produce first water based micro and nanodroplets and then, the liposomes were formed around them. Water solution droplets produced by atomization into an expanded liquid mixture formed by lipid compounds + ethanol + CO_2 were used. The basic idea is that lipids contained in the expanded liquid can spontaneously and rapidly organize in a layer around the water droplets in the high pressure vessel. Since the droplets of the water solution will be entrapped by the lipid layer, liposomes of controlled dimensions could be formed with high encapsulation efficiencies in the water pool located at the bottom of the precipitator.
Feasibility tests have been performed using phosphatidylcholine (PC) and phosphatidylglycerol (PG); process parameters were varied, to explore their effect on liposome size distribution and stability, to validate the process and to understand the mechanisms involved in liposomes formation. Preliminary encapsulation tests were also performed using bovine serum albumine (BSA) as a model protein, to verify the encapsulation efficiency of a water soluble compound.

MATERIALS AND METHODS

Materials
Soybean phosphatidylcholine (Soy PC) and phosphatidylglycerol (PG) was purchased from Lipoid (Ludwigshafen, Germany). Ethanol (≥ 99.5%) was obtained from Sigma-Aldrich (Milan, Italy) and CO_2 (>99.4% purity) was provided by SON (Naples, Italy). Trifluoroacetic acid (TFA 99%; Carlo Erba Reagents; Milan, Italy), Bovine serum albumin (BSA lyophilized powder ≥98%; Sigma-Aldrich; Milan, Italy) and HPLC grade

acetonitrile (Carlo Erba Reagents; Milan, Italy) were also used. All the compounds were used as received.

SuperLip apparatus layout

A schematic representation of the apparatus is depicted in Figure 1. It consists in three fed lines that deliver compressed CO_2 and ethanol solution to the saturator, and water to a high pressure chamber. CO_2 is pumped from a reservoir using a Lewa Ecoflow pump (mod. LDC-M-2, max. pressure 400 bar). Ethanol + phospholipids solution (0.16 $mgmL^{-1}$) and water were pumped by two different Gilson pumps (Model 305, Gilson FR). CO_2 and ethanol solution were continuous delivered to a saturator where the formation of the expanded liquid takes place. It is, then, injected by a capillary tube (8 cm length) inside of a high pressure chamber. Water was continuously atomized through a nozzle (80 µm diameter) inside of a high pressure vessel. This atomization step is important to produce small sub-micronic water that are the basis for the formation of an emulsion, when they contacted lipids contained in the ethanolic solution. Liposomes suspension is collected at fixed time intervals into a reservoir located downstream the high pressure vessel using an on-off valve.

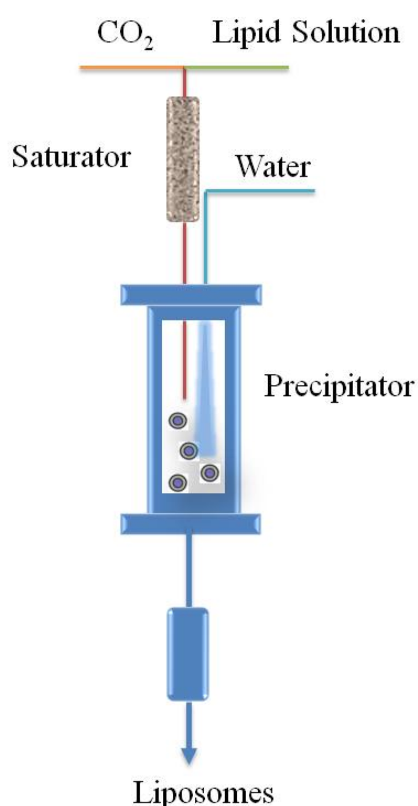

Figure 1: Schematic representation of the SuperLip process layout.

Liposomes characterizations

The mean size (MD), polydispersion index (PDI) and zeta potentials of the liposomal preparations were measured by Laser Diffraction analyzer (DLS; Zeta Sizer Malvern Inc., Wocherstershire, UK) equipped with a 5.0 mW He-Ne laser operating at 633 nm. Measurements were made in at a temperature of 25 ºC and scattering angle of 173°.

Liposomes were observed at a field emission scanning electronical microscope (FESEM LEO 1525, Carl Zeiss SMT AG). Samples were prepared by adding droplets of the

colloidal suspension over aluminium stubs, air-dried and sputter-coated with chrome (Agar Scientific, Stansted, UK). The amount of BSA loaded into the liposomes was measured by HPLC (model 1200 series; Agilent Technologies Inc., Italy) equipped with a LiCrosphere C18 column (250 mm × 4.6 mm), packed with 5 μm particles of 100 Å pore size, according to methods previously reported [16].

RESULTS

Pressure and temperature parameters

In the SuperLip process the principal step is the atomization of water droplets in the high pressure vessel filled with an expanded liquid mixture of lipid compounds + ethanol + CO_2.
Indeed, thanks to favorable interaction of phospholipids with water droplets a very fast formation of a lipidic layer around the atomized water based droplets is expected, forming w/CO_2 micelles. These layered droplets, continue their flight and fall in the water pool located at the bottom of the precipitator, where the process of liposomes formation is completed as consequence of interaction with the receiving external water solution, i.e. w/w emulsion is formed. The first set of experiments was performed to form empty PC liposomes, and therefore, pure water was atomized. Temperature inside the vessel and the mixer, was set at 40°C and vessel and mixer pressure were varied from 100 to 150 bar. The nozzle used for atomization had 80 μm internal diameter and water flow rate was fixed at 10.0 mL/min. The w/w gas/liquid ratio (GLR) in the saturator was fixed at 2.42 with the following flow rates: 6.7 g/min for the CO_2, 3.5 mL/min for phospholipids ethanolic solution. Considering the high pressure phase equilibra data for the system CO_2-ethanol-water at 40°C and 100-200 bar pressure range, reported in Figure 2, the operative conditions selected ensure that the operative point, reported in the same figure, is located inside the immiscibility hole where water and expanded liquid phase are not miscible and a w/CO_2 emulsion can be formed.
Table 1 reports the mean diameter of the liposomes produced by SuperLip in these first experiments, operating at different pressures. Liposomes mean diameter varied between 264 (±66) and 130 (±62) nm when the pressure was increased from 100 and 150 bar. The effect of pressure on liposomes mean size can be explained with an increase of CO_2 density and consequently of expanded liquid mixture density [17] (pure CO_2 density data under pressure are reported in Table 1, calculated using the Bender equation of state [18]) that favors atomization of water injected in the high pressure vessel [19]. The generation of smaller water droplets, consequently, leads to the decrease of liposomes diameter. A FESEM image of liposomes morphology obtained at 100 bar, 40°C, GLR 2.42 is reported in Figure 3.

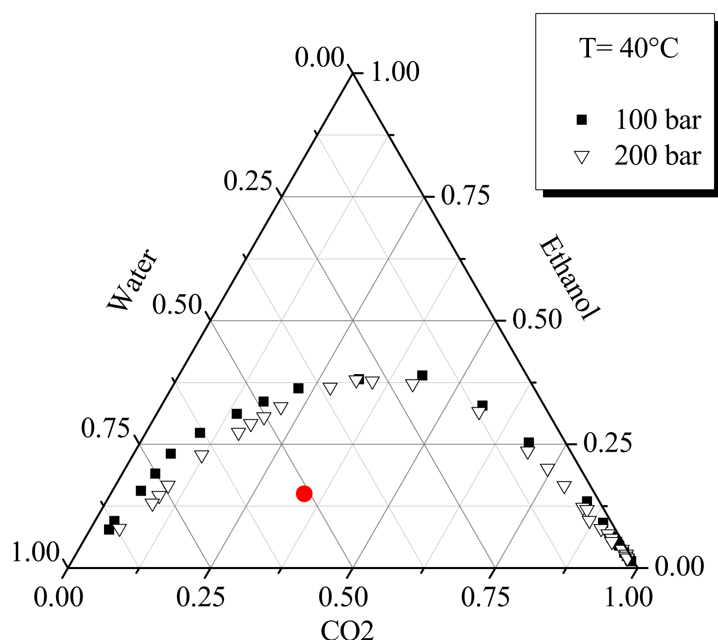

Figure 2. Phase equilibria data for the CO_2-ethanol-water system at 40°C and 100÷200 bar pressure range, adapted from [20]. The position of the operative point for the experiments conducted at different pressure is also reported (red colored).

Mixer & Vessel p(bar)	CO_2 density ($kg.m^{-3}$)	Mean size (nm ± SD)
100	625	264±66
125	730	174±69
150	815	130±62

Table 1. Liposomes size distribution. Process conditions: 40°C, CO_2 flow rate 6.7 g/min, GLR= 2.42, water flow rate 10 mL/min, nozzle diameter 80 µm.

The same set of experiments at 40°C and at different precipitation vessel pressure were performed using a mixture of PC and PG in the weight ratio 9/1. In this case larger liposomes have been obtained, indeed liposomes mean diameter varied between 442 (±110) and 209 (±72) nm when the pressure was increased from 100 and 150 bar. The effect of the increasing pressure is again of reducing liposomes dimensions. A comparison of results obtained with the same process conditions using only PC and PC/PG mixture is reported in Figure 4. From this figure it appears more evident that when PG is used in the formulation of the lipid bilayer vesicles with larger dimensions were obtained systematically, instead the effect of pressure is confirmed as the same found using only PC.

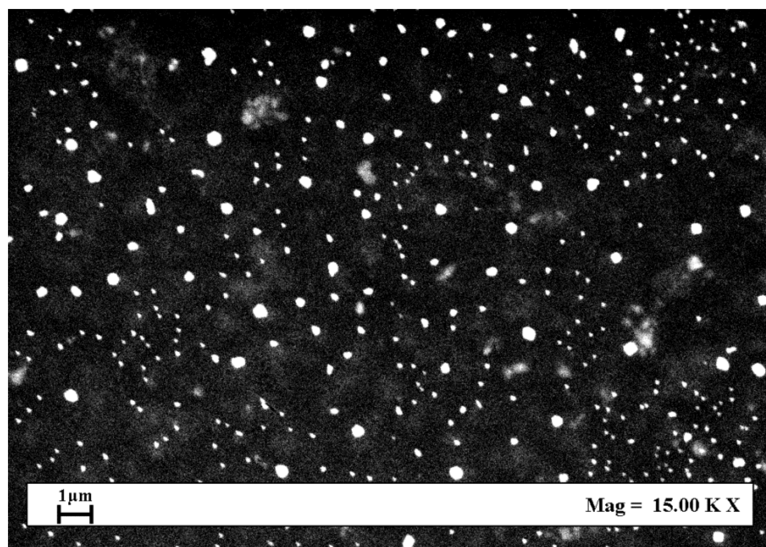

Figure 3. SEM image of PC liposomes produced using SuperLip at 100 bar, 40°C, GLR 2.42.

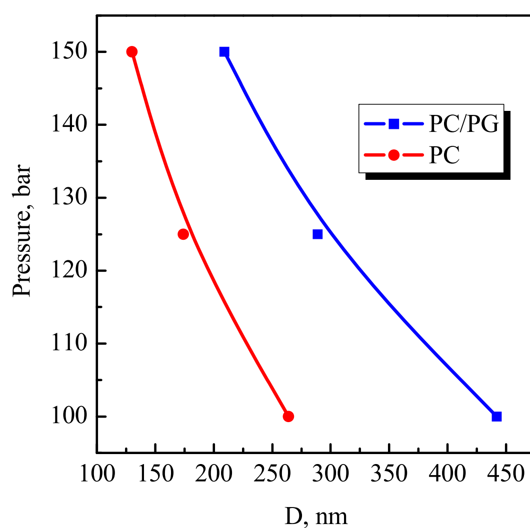

Figure 4. Effect of pressure and at 40°C for PC and PC/PG liposomes diameter.

Protein encapsulation

To demonstrate the potential improvement of encapsulation efficiency of hydrosoluble drugs using *SuperLip* process, some experiments were performed loading BSA in the water phase to be atomized and the encapsulation efficiency (EE) of the formed PC liposomes was measured. The theoretical loading was 1.7 % w/w of PC weight, corresponding to the composition of the water solution injected in the precipitator. Experiments were performed (in triplicates) at the operative conditions of: nozzle diameter 80 µm, 125 bar, 40 °C, GLR=2.42. Liposomes with an average diameter of 193±77 nm were obtained. This result is in good agreement with the

one reported in Table 1 for the analogous experiment performed using pure water: indeed, the mean liposomes diameter only slightly increases in the case of water loaded with BSA, as a consequence of an increase of cohesive forces during atomization, due to the presence of the solute. The encapsulation efficiency obtained during these tests ranged between 85 and 90%, confirming the hypothesis of a very high efficiency of the entrapment of hydrophilic compounds, thanks to the direct formation of liposomes around the water solution containing BSA.

CONCLUSION

The *SuperLip* process proposed in this work, has the advantage, with respect to the other techniques proposed in the literature, of a continuous layout and the water phase is directly atomized in the high pressure vessel. This process allows a spontaneous organization liposomes in the high pressure vessel, showing an unimodal submicronic PSD. The fact that the liposomes are formed on the fly allows higher encapsulation efficiency, because the active compound contained in the water solution is efficiently entrapped in the formed vesicles.
Further studies are needed to investigate more complex loading of liposomes with different kind of active compounds.

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