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Liposomes preparation using a supercritical fluid assisted continuous process

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HIGHLIGHTS

- A new supercritical assisted process for liposomes production is proposed.
- Liposome suspensions with diameters ranging between 120 and 300 nm are obtained.
- An interpretation of liposome formation mechanism is attempted.

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ABSTRACT

Liposomes are formed by phospholipids that spontaneously generate bilayers vesicles as a consequence of their interactions with water; they can be very efficient drug carriers, capable to preserve the activity and/or improve the safety of several therapeutic molecules. In this paper, a new continuous supercritical fluid process, named Supercritical Assisted Liposome formation (SuperLip), is proposed to prepare liposomes of controlled submicrometric size. In this process, water droplets are produced by atomization inside an 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, forming a w/CO_2 emulsion and liposomes (w/w emulsion) are formed when they fall in the water pool located at the bottom of the vessel. Experiments have been performed varying process operating parameters like pressure, temperature and flow rate ratio between CO_2 and ethanol, producing liposomes of different size and distribution ranging between 130 ± 62 and 294 ± 144 nm. The results demonstrated that atomized liquid droplets are transformed efficiently into submicronic liposomes as a consequence of the spontaneous organization of the vesicles on the fly in the high pressure vessel.

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 atomized water phase was efficiently entrapped in the formed vesicles.

1. Introduction

Liposomes are formed when phospholipids spontaneously self assemble into vesicles in the presence of water, producing microscopic aqueous droplets surrounded by a lipidic membrane [1]. They can be also defined as water in water 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. Liposomes diameter can range from about 100 nm to several microns [2]; they can be used as drug carriers or to improve drug bioavailability [3–5].

Common 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 [6,7].

In the field of particle formation and production of delivery vehicles, supercritical fluid technologies can overcome several limitations of conventional processes, such as the extensive use of organic solvents, high operating temperatures and mechanical stresses that can degrade labile compounds. Moreover, supercritical fluid technologies can offer a better control over the morphology of products at micrometric and nanometric scale; therefore,

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supercritical fluids have been proposed as solvents, antisolvents, and processing media in many processes related to pharmaceutical and biomedical compounds [8–14]. Recently, some techniques based on the use of supercritical CO_2 ($scCO_2$) have been proposed also for liposomes preparation [2,15–19]; they tried to take the advantage of the enhanced mass transfer of supercritical fluids [20] and can be roughly divided in two categories: (a) *two steps processes* in which the dried lipid particles need to be, then, rehydrated [18,21–25]; (b) *one step processes* in which a liposome water suspension is directly obtained at the end of the process [26].

Two steps processes use a phospholipid organic solvent solution (as a rule in ethyl alcohol), that is continuously sprayed into supercritical CO_2, used to extract the organic solvent. This contact leads to a rapid supersaturation of the solution that causes the fast nucleation and consequent, formation of dried lipid particles [9,27]. Phospholipidic particles have to be subsequently rehydrated to produce liposomes. This kind of processes has some drawbacks related to the control of particles dimension and distribution and also shows very low encapsulation efficiencies (ranging around 10–20%) [28]. Indeed, the solution used to rehydrate liposomes also contains the drug to be encapsulated and only a small part of it is effectively entrapped in the lipidic bilayer.

In the *one steps processes*, hydration of liposomes occurs under pressure [17,29] or during the depressurization step [16,20]. Particularly, Otake et al. [17], developed a process named Supercritical Reverse Phase Evaporation (scRPE) in which phospholipids are mixed under constant stirring with $scCO_2$ and ethanol (used as co-solvent) in a variable volume cell, operated at constant pressure and temperature, at values usually higher than the lipids phase transition. After the equilibrium is reached, water is slowly introduced into the system and, then, the pressure is rapidly released. Liposomes with diameters ranging from 0.1 to 1.2 μm were obtained, with an encapsulation efficiency of 25% for glucose, in the water phase, and 63% for cholesterol, in the organic phase. The same authors [29] also reported a different process, derived from the scRPE, that produces inhomogeneous mixtures of phospholipids and aqueous solution (named IscRPE) into carbon dioxide, using a variable volume cell magnetically stirred. Liposomes with a mean diameter of 1.5 μm were formed in this case. scRPE and IscRPE processes have the advantage of producing liposomes in one step; but, they still have a batch layout and do not guarantee a good control of size and distribution of liposomes. Indeed, they use a decompression step from supercritical conditions to room conditions to produce the strong mixing of the lipids and water phase, that promotes liposomes formation [26]; the low reproducibility of the decompression/mixing process can generate liposomes with different batch to batch size characteristics.

Meure et al. [20] reported another process named *Depressurization of an Expanded Solution into Aqueous Media* (DESAM). In this case, the phospholipids are initially dissolved in an organic solvents and, then, CO_2 is added to the system to obtain an expanded lipid solution that is atomized through a nozzle into a heated aqueous medium, at room pressure. Frederiksen et al. [16] reported a similar, *one step* process, with the direct generation of a phospholipids/organic solvent/CO_2 mixture into a high pressure vessel, that was, then, expanded into a water phase. The liposomes produced using the depressurization of an expanded liquid mixture in a water solution, showed a bimodal distribution with mean diameters of 50 and 250 nm. Furthermore, encapsulation efficiency studies were not performed; considering that liposome were formed in a water bath, in which the drug should be dissolved, the expected encapsulation efficiency is low, because only a small part of the water solution will be entrapped by the lipidic layer; whereas, the remaining major part of the solution will remain in the continuous phase.

Considered the limitations indicated for the previous processes, in this work a new 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. SuperLip feasibility tests are performed and process parameters are varied, to explored their effect on liposome size distribution and stability, to validate the process and to understand the mechanisms involved in liposomes formation. Preliminary encapsulation tests are also performed using bovine serum albumine (BSA) as a model protein, to verify the encapsulation efficiency of a water soluble compound.

2. Materials, methods and apparatus

2.1. Materials

Soybean phosphatidylcholine (Soy PC) 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). Distilled water was used throughout all the formulations. 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.

2.2. SuperLip apparatus layout

A schematic representation of the SuperLip apparatus is reported in Fig. 1. It consists of three feed lines that deliver compressed CO_2

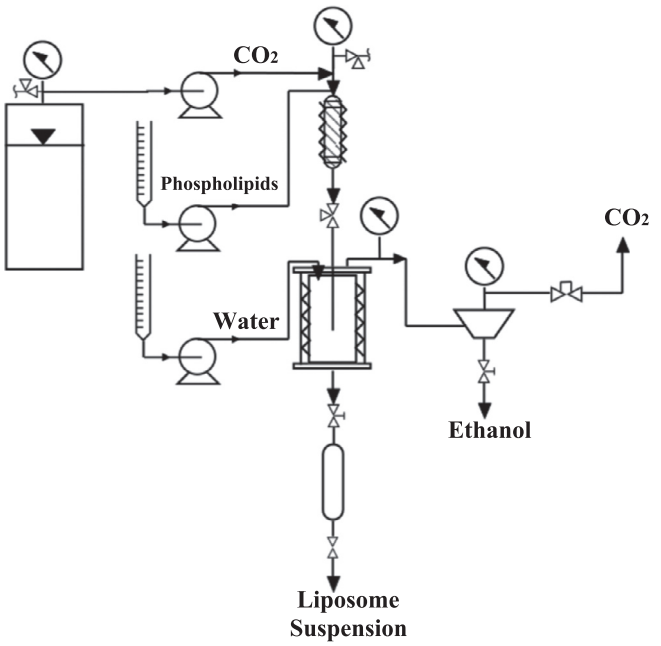

Fig. 1. Schematic representation of the SuperLip process layout.