Journal of $CO_2$ Utilization 18 (2017) 181–188

Contents lists available at ScienceDirect

Journal of $CO_2$ Utilization

journal homepage: www.elsevier.com/locate/jcou

# Supercritical $CO_2$ assisted liposomes formation: Optimization of the lipidic layer for an efficient hydrophilic drug loading

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ARTICLE INFO

*Article history:*
Received 10 November 2016
Received in revised form 17 January 2017
Accepted 1 February 2017
Available online 13 February 2017

*Keywords:*
Liposomes
Supercritical fluids
Theophylline
Cholesterol
Phosphatidylethanolamine

ABSTRACT

Liposomes are natural vesicles generally based on phosphatidylcholine (PC). The optimization of the lipid bilayer composition with the addition of little percentages of natural lipids is still at early stage due to the difficulties experienced by classical liposome formation processes, mainly, in reproducibility and encapsulation efficiency.

Supercritical assisted liposome formation (SuperLip) has demonstrated that these limitations can be overcome. Therefore, in this work, this process has been tested to produce liposomes of controlled nanometric diameter and the effect of water solution flow rate on drug encapsulation efficiency was investigated. The addition of cholesterol (Chol) or phosphatidylethanolamine (PE) was also studied to gain the control on the release rate of the drug entrapped in liposomes. Theophylline was selected as the model hydrophilic drug.

Using SuperLip process, PC/Chol and PC/PE liposomes were successfully produced with nanometric mean diameters down to 200 nm. Optimization of both lipid composition and SuperLip operative parameters allowed to obtain theophylline encapsulation efficiencies up to 98%. Drug release kinetics were affected by liposome composition, in particular, the addiction of Chol and PE allowed to slow down theophylline release rate. These results confirmed the possibility of producing liposomes with a complex architecture of the lipid membrane using the SuperLip process.



## 1. Introduction

Liposomes are lipidic vesicles of micrometric or nanometric dimensions, characterized by one or more double layers of phospholipids. Liposome formation process is spontaneous, since phospholipids are able to self-assemble into spherical hollow structures in the presence ofl water, to reach a more favorable energetic state. The closure of the lipidic double layer takes place in successive steps. First lipidic bilayer is planar and flat that corresponds to an unfavorable energetic state. Then the angles of the bilayer plane start to fold like a balloon as a consequence of the interaction with water. Finally, lipidic double layer folds completely, separating the inner water volume by the external one, assuming a spherical shape [1–3].

It is possible to encapsulate either hydrophilic drugs in the inner aqueous compartment, either lipophilic compounds in the lipidic double layer. Lipidic double layer is ideal for drug delivery to the human body, due to the similarity with natural cells [4,5].

Liposomes are not toxic and biodegradable drug delivery systems. Indeed, their lipidic double layer is able to fuse with human cell membrane, becoming part of it (direct cellular up-take) [6]. Vesicles composed of natural phospholipids are also biologically inert if compared to synthetic ones, and this makes them more stable over time [7]. Lipid vesicles are able to preserve drugs from degradative phenomena. Moreover, the controlled release can ensure a constant drug concentration level in the human body. Furthermore, liposomes of nanometric dimensions (below 200 nm [8]) can circulate in the blood stream without being recognized by macrophages; they can easily penetrate into small interstices of tumoral tissues where they can sustain the drug release over days or even weeks [9–11]. In particular, liposomes with nanometric dimensions can avoid sedimentation and blockage of blood circulation [12] and are easily sterilizable being smaller than bacteria average size [13].

*Abbreviations:* PC, phosphatidylcholine; PE, phosphatidylethanolamine; Chol, cholesterol; MD, mean diameter; SD, standard deviation; PDI, polydispersion index; PSD, particle size distribution; DLS, dynamic light scattering; NTA, nanoparticles tracking analysis; EE, encapsulation efficiency; TE, trapping efficiency; WFR, water flow rate; TEM, transmission electron microscopy; SCF, supercritical fluid; SuperLip, supercritical assisted liposome formation.

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http://dx.doi.org/10.1016/j.jcou.2017.02.001
2212-9820/

For all these reasons, liposomes are largely studied drug delivery systems to encapsulate, control and protect the release of functional active principles in medical and therapeutic applications, in food, agricultural fields [14] and cosmetic industries [15].

The characteristics of liposomes are strictly related to chemical properties of phospholipids used during the preparation. The kind of lipids selected for liposome formation may modify the total surface charge of liposomes, the permeability, the encapsulation efficiency and the drug release [16–18]. The membrane solidity is often linked to the shape of phospholipids, particularly to the corner amplitude described by their two tails [19]. Phosphatidylcholine (PC) is the most commonly used phospholipid in liposomes membrane [14]. Cholesterol (Chol) is recognized to be compatible with the formation of vesicles. It is reported that it is able to prevent the formation of aggregates and has stabilizing effects. However, a high percentage of cholesterol in liposome formulation can cause liposome destabilization and the presence of Chol crystals in the aqueous bulk. The amount necessary to achieve stable carriers has not been clarified yet [20]. Phosphatidylethanolamine (PE) is another kind of phospholipid used in many cases for liposomes double layer formulations [21]. In this case, the main difference with PC tridimensional structure is the presence of a larger angle between the two lipid chains. PE general shape is truncated conical, while PC has a cylindrical shape [22]. Despite these interesting properties, a systematic study on the effect of PE or Chol incorporation in liposome membranes on vesicles mean diameter, drug encapsulation efficiency, drug release has not been performed yet. For example, some liposome formulations have been developed adding synthetic lipids to PE vesicles, obtaining nanoparticles but with low encapsulation efficiencies of active principles [23]. The effect of cholesterol incorporation was studied together with PEGylation or chitosan coating; but, focusing especially on the permeability effect on the membrane [24].

Methods for the preparation of liposomes have been developed in the last decades [25,26]. However, these methods present some drawbacks, as low reproducibility, batch operations, low encapsulation efficiency of hydrophilic compounds and a difficult control of liposome size distribution [27]. It is also difficult to remove the solvent from the final suspension, thus hindering the real industrial applications of these proposed technologies [28].

In the field of carrier production, supercritical fluid (SCF) technologies have been proposed to overcome several limitations of conventional processes for the production of micronized particles [29,30] carriers [31–34], coprecipitates [35–41] and nanocomposite polymeric structures [42–44]. Recently, some techniques based on the use of supercritical carbon dioxide ($CO_2$) have been proposed also for liposome production [45–49]. The advantages derived by the use of supercritical carbon dioxide are a larger diffusion coefficient of the lipids and a lower viscosity of the medium bulk. This brings to a better control of PSDs of liposomes produced. However, the supercritical fluids based processes for liposomes production have still some limitations related to the control of liposome dimension and size distribution and also show very low encapsulation efficiency of hydrophilic drug. The major limitation of these processes, both conventional and supercritical, derives from the hydration step of the lipid layer. Indeed, during this step, only a part of the water used for hydration is actually entrapped into liposomes, resulting in a low overall encapsulation efficiency.

Recently a supercritical technique, named SuperLip (Supercritical Assisted Liposome Formation) has been developed for the production of liposomes [50]. In this process first water droplets are produced, and then they are rapidly covered by phospholipids. Thanks to the high diffusion coefficient of $CO_2$, the lipids coverage is faster than in the conventional techniques for the production of liposomes. In this way it is possible to produce vesicles with a good control of particle size distribution and high encapsulation efficiency (EE). Indeed, some hydrophilic compounds have been encapsulated inside the aqueous core, with high encapsulation efficiencies up to 97% [51,52].

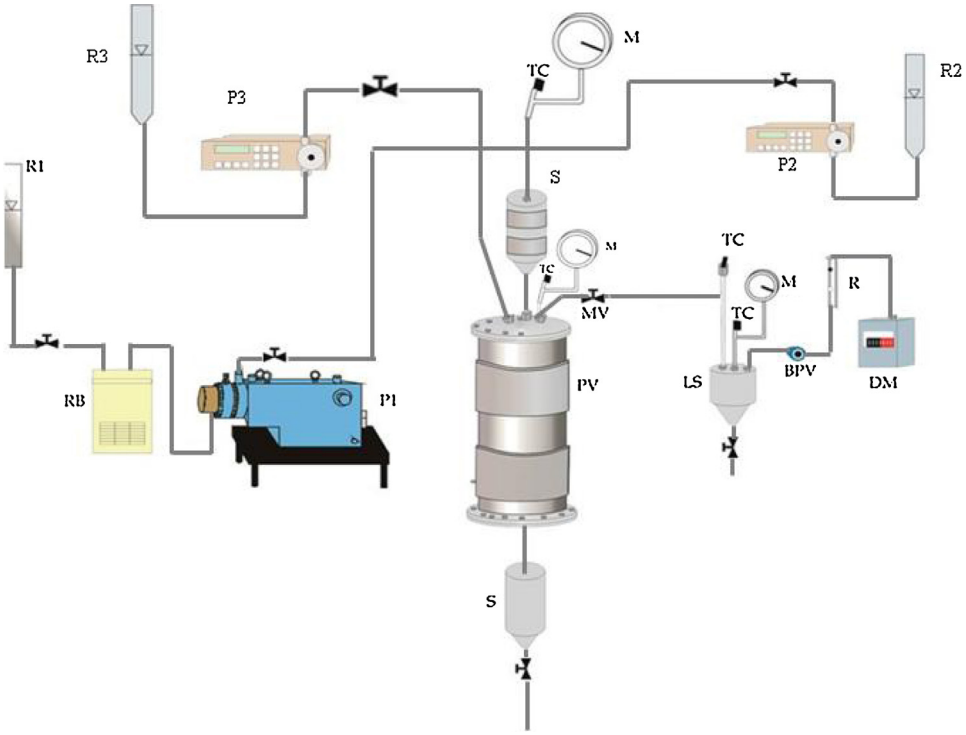


**Fig. 1.** Schematic representation of the SuperLip lab-scale plant. R1: $CO_2$ reservoir; R2: lipids reservoir; R3: water solution reservoir; RB: refrigeration bath; P1: $CO_2$ pump; P2: lipids solution pump; P3: water solution pump; S: expanded liquid saturator; PV: precipitation vessel; S: suspension recovery; LS: liquid separator; R: rotameter; DM: dry test meter; TC: thermocouple; M: manometer; MV: micrometric valve; BPV: back pressure valve.