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Efficient encapsulation of proteins in submicro liposomes using a supercritical fluid assisted continuous process

Roberta Campardelli[a], Islane Espirito Santo[a,b], Elaine Cabral Albuquerque[b], Silvio Vieira de Melo[b], Giovanna Della Porta[a], Ernesto Reverchon[a,*]

[a] Department of Industrial Engineering, University of Salerno, Via Giovanni Paolo II, 132, Fisciano, SA 84084, Italy
[b] PEI, Industrial Engineering Program, Department of Chemical Engineering, Federal University of Bahia, Rua Prof. Aristides Novis, 2, Federação, 40210-630 Salvador, BA, Brazil

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ABSTRACT

Recently a new supercritical assisted process was proposed in the literature for the production of liposomes, called Supercritical Assisted Liposome formation (SuperLip) process. It has been demonstrated that, using this new process, it is possible to produce liposomes based on phosphatidylcholine (PC) with a controlled particle size distribution. Also a preliminary encapsulation test revealed a high encapsulation efficiency of bovine serum albumin (BSA), used as model hydrophilic compound.

Being convinced of the great potential of the SuperLip process, in this work it has been studied the possibility of producing liposomes with a different composition of the bilayer membrane using the SuperLip process. Phosphatidylcholine (PC) and phosphatidylglycerol (PG) were used in a mixture and the production of liposomes was tested under different operative conditions: pressure, temperature and lipid composition. Results obtained using only PC and PC/PG mixture have been systematically compared. Furthermore a deepened study about the possibility to encapsulate BSA as model compound was performed. SuperLip experiments using different amount of BSA dissolved in the water internal phase were performed and the relative encapsulation efficiencies of produced liposomes were estimated. A comparison of encapsulation efficiency of liposomes produced, at the same BSA loadings, using a conventional preparation process (Bangham method) was also performed. The stability of entrapped protein was investigated.

Sub-micrometric liposomes of soybean phosphatidylcholine (PC) of different size and distribution ranging between 250 ± 58 nm and 330 ± 82 nm were successfully produced. When using PG coupled with PC, larger liposomes were produced, ranging between 280 ± 70 nm and 350 ± 101 nm. Both PC and PC/PG liposomes were stable over one month, thanks to the large and negative surface charge (zeta potential ranging between -20 mV and -30 mV).

For the drug encapsulation tests different BSA theoretical loadings with respect to the lipid amount (10–30–60%, w/w) were tested. In the case of SuperLip process, very high encapsulation efficiencies (92–98%) were obtained at all the drug loadings; lower encapsulation efficiencies were instead obtained using the Bangham method (2–57%). Results reported in this work demonstrated that using SuperLip process, the protein contained in the water phase can be efficiently entrapped without damaging the protein structure as confirmed by FTIR analysis of processed BSA.

1. Introduction

Liposomes are vesicles in which a small aqueous volume is surrounded by a bilayer membrane, normally composed of phospholipids [1]. Due to their similarities with natural cells, liposomes have been reported as ideal drug carriers [2]. They have been investigated for the delivery of chemotherapeutic agents for cancer [3], therapeutic proteins for cell signaling [4], vaccines for immunological protection [5], radiopharmaceuticals for diagnostic imaging [6], and nucleic acid-based medicines for gene therapy [7]. Despite the recognized importance of liposomes in pharmaceutical and biomedical field, these carriers are being introduced with difficulties in the market. This fact is partly due to problems related with the low encapsulation efficiency, repeatability of the

* Corresponding author.
E-mail address: ereverchon@unisa.it (E. Reverchon).

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batch-to-batch processing and scale-up of conventional preparation methods.

The starting point for all conventional methods of liposome production is the dissolution of phospholipids in an organic solvent, and the main difference between these methods is the way in which the lipid membrane is dispersed in the aqueous medium [8–10]. These methods show some drawbacks, such as the large number of steps needed to produce the vesicles, the utilization of a large amount of organic solvents during the process, the difficulty in reproducing liposome diameter, the low stability of produced liposomes and low encapsulation efficiencies [11]. Furthermore, in the case of proteins encapsulation, these processes can lead to a rapid denaturation of the protein with loss in its functionality [12].

In the field, of particles formation and carriers production, supercritical fluid technologies have been proposed to overcome several limitations of conventional processes, such as the control of particle size at micrometric and nanometric level, the extensive use of organic solvents, high operating temperatures and mechanical stresses that can degrade labile compounds [13–17]. Recently, some techniques based on the use of supercritical CO_2 ($scCO_2$) have been proposed also for liposome preparation [18–23]; they try to take the advantage of the enhanced mass transfer of supercritical fluids [24] and can be divided in two categories: *two steps processes* in which the dried lipid particles need to be rehydrated [11,22,25–28], and *one step processes* in which a liposome-water suspension is directly obtained at the end of the process [29–31]. These processes have still some limitations related to the control of liposome dimension and distribution and also show very low encapsulation efficiencies, especially for liposomes containing hydrophilic drugs. For example, water soluble markers, such as fluorescein isothiocyanate–dextran and zinc phthalocyanine tetrasulfonic acid, have been entrapped into liposomes of about 200 nm using the supercritical liposome method. This method consists of two main steps: the high-pressure part, in which the lipid components are dissolved under pressure in supercritical carbon dioxide, and the low-pressure part, in which the homogeneous supercritical solution is expanded and simultaneously mixed with the aqueous phase to produce liposomes encapsulating the water soluble drug. Encapsulation efficiency reported was of about 20% [32]. Docetaxel, a chemotherapeutic agent, was entrapped in liposomes of about 270 nm with an encapsulation efficiency of 37% using a supercritical antisolvent method, in which solid lipid particles were formed and then hydrated with a water solution containing the drug [33].

These examples show that supercritical fluids based processes, share with the conventional ones, low encapsulation efficiency of hydrophilic drugs, substantially due to the fact that only a part of the water used to hydrate the lipids is entrapped in the lipid membrane.

Reverchon and co-workers recently proposed a supercritical fluid based process for liposome production, named Supercritical Assisted Liposome formation (SuperLip) [34]. Differently from the previously proposed techniques, the basic principle of this process is to produce first water based micro and nanodroplets and, then, the liposomes are formed around them. Water solution droplets are produced by atomization into a mixture formed by lipid compounds + ethanol + CO_2 forming an expanded liquid; i.e., an organic solvent that is liquid at room temperature and pressure, modified by dissolution of a dense CO_2 that changes the diffusivity and the surface tension of the liquid compound, while maintaining part of its original characteristics, such as the solvent power. The idea is that lipids contained in the expanded liquid can spontaneously organize in a layer around the water droplets in the high pressure vessel and a water in CO_2 emulsion is formed. At the end of this process, droplets fall in the continuous water pool located at the bottom of the vessel and a water in water emulsion is formed; i.e., liposomes of controlled dimension could be formed with high encapsulation efficiencies. Liposome suspension produced using SuperLip process can be purified from ethanol residues using a supercritical method, as reported in a recent publication [35].

In the previous paper [34], the SuperLip process was successfully applied for the production of liposomes using phosphatidylcholine (PC) as phospholipids. The effect of different operating parameters (pressure, temperature and nozzle diameter for the atomization of water) was studied on the production of empty liposomes. Also a first encapsulation test was performed on a model compound, bovine serum albumin (BSA), as a proof of concept of loaded liposome formation. Results obtained confirmed a potential of this process in the field of liposomes production.

For these reasons, the aim of this work is the application of SuperLip process for the production of liposomes with a more complex lipidic structure: a series of experiments for the production of liposomes formed by phosphatidylcholine (PC) and phosphatidylglycerol (PG) mixture will be proposed. The production of liposomes will be tested under different operative conditions: pressure, temperature and lipid composition. A systematic comparison between the results obtained using only PC and PC/PG mixture will be performed. Furthermore, the study of the possibility to encapsulate therapeutic thermosensitive compounds will be systematically performed, using BSA as model compound. For this reason, experiments using different amounts of BSA dissolved in the water atomized in the high pressure vessel will be performed and the relative encapsulation efficiencies of produced liposomes will be measured. A comparison of the encapsulation efficiency of liposomes produced, at the same BSA loadings, using a conventional preparation process (Bangham method) will also be performed. The stability of entrapped protein will be investigated.

2. Materials, methods and apparatus

2.1. Reagents

Soybean phosphatidylcholine (PC) and phosphatidylglycerol (PG) were 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 in all formulations. Bovine serum albumin (BSA), lyophilized powder ≥98%; was provided by Sigma-Aldrich (Milan, Italy). All the compounds were used as received.

2.2. SuperLip apparatus layout

Liposomes were prepared using the SuperLip apparatus. It consists of five principal parts, as also represented in Fig. 1: Saturator: in this part of the plant the formation of the expanded liquid, formed by ethanol–CO_2–phospholipids, takes place. Then, the expanded liquid is fed to the precipitation vessel. Atomization: a nozzle located at the top of the precipitation vessel allows the formation of water droplets. Precipitation vessel: in this part of the apparatus the droplets come in contact with the expanded liquid formed in the saturator. Suspension recovery: the produced suspension is withdrawn at fixed time intervals. Separator: the solvent, used for the expanded liquid formation, is recovered after depressurization.

The apparatus consists of three different lines for the delivery of CO_2, water and ethanol–phospholipids solution respectively. CO_2 is taken from a reservoir and delivered to the saturator using a membrane pump (Lewa Eco model LDC-M-2, Germany). The ethanolic solution and the water phase are pumped using two different pumps (Gilson model 305, France).

The ethanol solution and CO_2 are continuously fed to the stainless steel saturator (Swagelok, 150 cm^3, Pmax 5000 psig, USA), at a fixed gas to liquid ratio (GLR). The saturator is heated using band