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

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ABSTRACT

In this work a recently developed supercritical fluid based process, called Supercritical Assisted Liposome formation (SuperLip) has been successfully applied to the production of submicrometric liposomes of Phosphatidylcholine (PC) and phosphatidylglycerol (PG) for the encapsulation of bovine serum albumin (BSA).
The effect of operating parameters such as pressure, temperature and lipid composition have been tested and results obtained using only PC and PC/PG mixture have been systematically compared. Sub-micrometric liposomes of soybean phosphatidylcholine (PC) of different size and distribution ranging between 250 ± 58 nm and 330 ± 82 nm were successfully produced. Indeed, 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 -20mV and -30 mV).
Drug encapsulation tests were performed using bovine serum albumin (BSA), used as a model therapeutic protein. Different BSA theoretical loadings with respect to the lipid amount (10-30-60%, w/w) were tested. BSA loaded liposomes have been also produced using the conventional Bangham method for comparison purposes. In the case of SuperLip process very high encapsulation efficiencies (92-98%) were obtained at all the drug loadings; instead, lower encapsulation efficiencies were 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 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 compressed gas to modify 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 recently 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 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 the hypothesized mechanism of liposome formation.

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 experiment for the production of phosphatidylcholine (PC) in a mixture with phosphatidylglycerol (PG) liposomes will be proposed. A systematic comparison between the results obtained using different lipid composition and changing operating parameters will be conducted. Furthermore, deepened study about the possibility to encapsulate therapeutic thermosensitive compounds will be performed, using BSA as model compound. For this reason, experiments using different amount of BSA dissolved in the water atomized in the high pressure vessel will be conducted and the relative encapsulation efficiencies of produced liposomes will be estimated. A comparison of encapsulation efficiency of liposomes produced, at the same BSA loadings, using a conventional preparation process (Bangham method) will also be performed. In conclusion 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 **Figure 1**:

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 in continuous to the precipitation vessel.
2. Atomization**:** a nozzle located at the top of the precipitation vessel allows the formation of water droplets.
3. Precipitation vessel: in this part of the apparatus the formed droplets come in contact with the expanded liquid formed in the saturator.
4. Suspension recovery: the produced suspension is accumulated during the experiment and withdraw at fixed time intervals.
5. Separator: the solvent used for the expanded liquid formation is recovered after depressurization.

In details 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 Gilson pumps (model 305, Gilson, France).

The ethanol solution and CO_2 are continuously fed to the stainless steel saturator (model Compliant Sample Cylinder, 150 cm^3, Pmax 5000 psig, Swagelok, USA), at a fixed gas to liquid ratio (GLR). The saturator is heated using Watlow Thinband (model STB1R1A1-L12 240 V, 140 W, Italy). The saturator contains stainless steel packing (ProPak, 1889 m^{-1} specific surface, 0.94 void degree, Scientific Development Company, Pennsylvania). The packing elements allow an intimate mixing between the ethanol solution and CO_2, producing the expanded liquid, that is then delivered to the precipitation vessel. In the same vessel water (or a water solution in the case of loaded liposomes) is atomized through a nozzle of 80 µm diameter. The atomization and the delivery of the expanded liquid are obtained in equi-current. The precipitation vessel is a high pressure stainless steel cylinder, with an internal volume of 1600 cm^3. It is thermally heated using Watlow heating bands (model MB03J2AE-3007 240 V, 800 W, Watlow, Italy). At the bottom of the precipitation vessel a smaller stainless steel cylinder (150 cm^3) is located and used to allow the accumulation of the produced suspension during the experiment. The suspension is withdraw at fixed time intervals using an on-off valve. A separator is located downstream the precipitation vessel and is used to recover CO_2 and ethanol after depressurization. The separator is a stainless steel vessel of 330 cm^3 internal volume, in which the pressure is regulated using a back-pressure valve (model 26-1723-44, Tescom, Italy). A micrometric valve is also located on the CO_2 vent line for a fine regulation of the pressure in the precipitation vessel. A rotameter and a dry test meter are used to measure the CO_2 flow rate [34].

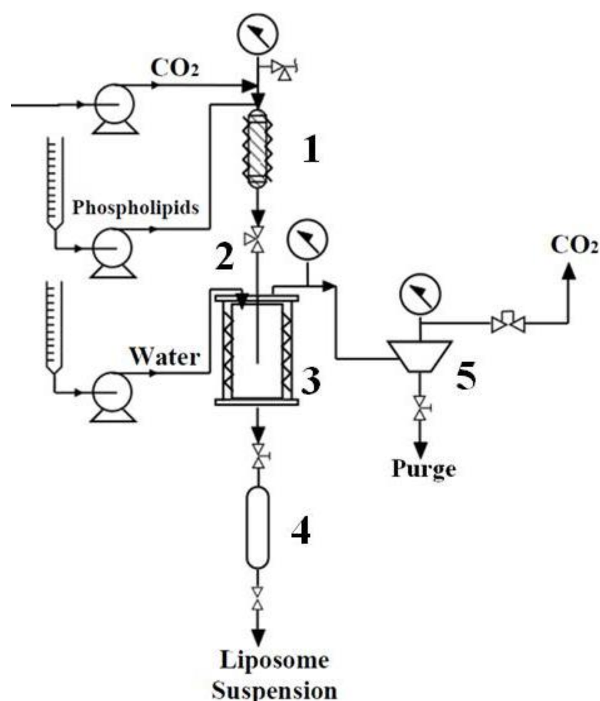


Figure 1: Representation of the SuperLip process layout.

In this paper also some experiments of liposome production using the Bangham method have been performed, for comparison purpose. In these cases, a fixed amount of phospholipids was dissolved in 100 mL of Chloroform. The lipid solution was then put in a round flask and the solvent was evaporated under reduced pressure, to produce a thin lipid layer. Then, 100 mL of water or a BSA water solution were added and the system was magnetically stirred at 400 rpm for 30 minutes to allow liposome formation.

2.3 Liposome characterization: morphology, size distribution and protein assay

The morphology of produced liposomes was studied using a Field Emission-Scanning Electron Microscope (FE-SEM mod. LEO 1525; Carl Zeiss SMT AG, Oberkochen, Germany). Samples were prepared using the following procedure: a drop of liposome suspension was placed over an adhesive Carbon tab previously stuck over an Aluminum stub. The drop was left dry at air over night. The sample was, then, covered with Gold using a sputter coater, thickness 250 Å (model B7341; Agar Scientific, Stansted, UK).

Particles size distribution (PSD), mean diameter (MD), standard deviation (SD), polidispersity index (PDI) and zeta potential of the liposome were measured using a Malvern Zeta Sizer laser scattering instrument (mod. Zetasizer Nano S, Worcestershire, UK).

To determine encapsulation efficiencies [36], liposome suspension was centrifuged at 6500 for 45 minutes. Then, the concentration of the drug in the water supernatant (mg_{drug}) was analyzed using UV-vis spectroscopy, using a wave length of 280 nm for BSA. The encapsulation efficiency (EE) was calculated with respect to the theoretical drug content (mg_{loaded}) using the following equation:

$$EE = 100 - \left(\frac{mg_{drug}}{mg_{loaded}}\right) * 100$$

Fourier transform infrared (FT-IR) spectra were obtained via M2000 FTIR (MIDAC Co, Costa Mesa, CA), at a resolution of 0.5 cm^{-1}. The scan wavenumber range was 4000-400 cm^{-1}, and 16 scan signals were averaged to reduce the noise.
The solution spectra were collected using a 5.4 m path length liquid cell with CaF_2 windows.

3 RESULTS AND DISCUSSION

3.1 Production of empty liposomes: effect of temperature, pressure and lipids composition

In the first set of experiments we produced empty PC liposomes, atomizing pure water. Temperature inside the vessel and the mixer, was set at 40 °C and vessel and mixer pressure were set at 125, 150 or 175 bar. The nozzle used for atomization had 80 µm internal diameter and water flow rate was set at 10.0 mL/min. The gas/liquid ratio (GLR w/w) in the mixer was fixed at 2.42 with the following flow rates: 6.7 g/min for CO_2, 3.5 mL/min for phospholipids ethanol solution. PC concentration in ethanol solution was fixed at 5 mg/mL. Considering the high pressure phase equilibria data for the system CO_2-ethanol-water at 40 °C in the 100-200 bar pressure range, (reported in the **Figure 2** and adapted from [37]), the process conditions we selected, ensure that the operative point, represented in the same figure as a red dot, is located inside the miscibility hole where water and expanded liquid split in two phases and a water in the organic CO_2 solution (ethanol + PC) emulsion can be formed.

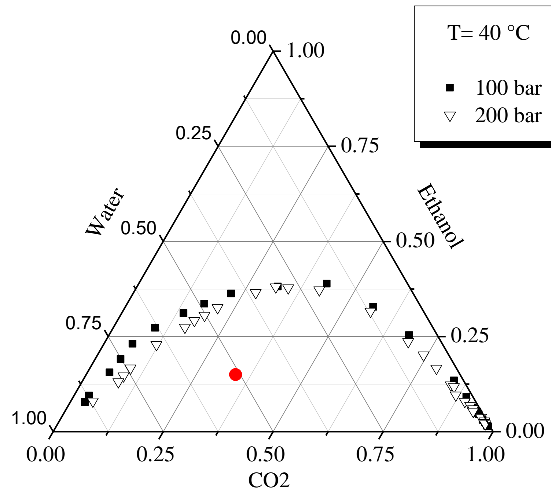


Figure 2. Phase equilibrium data for the CO_2-ethanol-water system at 40 °C, at 100 and 200 bar, adapted from [37]. The position of the operative point of the experiments performed in this study is reported (red dot).

Table 1 reports the mean diameter of PC liposomes produced by SuperLip in these experiments at different pressures. Liposome mean diameter varied between 330 (±82) and 250 (±58) nm when the pressure was increased from 125 and 175 bar. Increasing the pressure a sharpening of the PSD was observed: smaller PDI were obtained in correspondence of the higher pressures. A comparison of the PSDs of liposome suspensions obtained at different pressures is reported also in **Figure 3**, where the effect of pressure on liposome mean diameter can be appreciated.

The effect of pressure on liposome mean size can be explained considering that, increasing the pressure, an increase of CO_2 density and, consequently, of expanded liquid mixture density is obtained [38, 39], that favors atomization of the water injected in the high pressure vessel [40]. The generation of smaller water droplets, consequently, leads to the formation liposomes with smaller diameter.

Table 1. Liposome size distributions data obtained at 40 °C and 70 °C, operating at different pressures.

	T_{mix} 40 °C				**T_{mix} 70 °C**	
p(bar)	**PC MD ± SD nm**	**PDI**	**PC/PG MD ± SD nm**	**PDI**	**PC/PG MD ± SD nm**	**PDI**
125	330±82	0.25	350±101	0.29	484±150	0.31
150	280±68	0.24	300±75	0.25	432±120	0.28
175	250±58	0.23	280±70	0.25	343±92	0.27

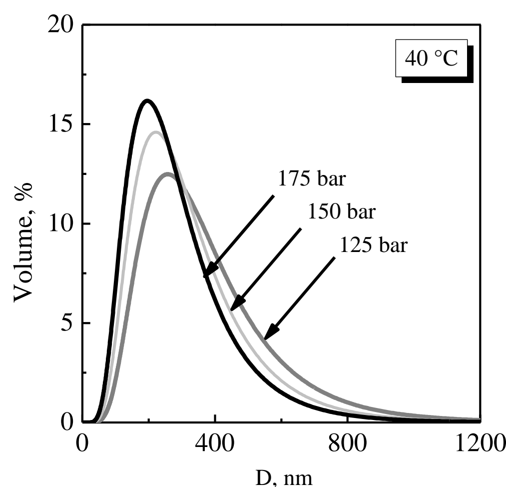


Figure 3. PSDs of PC liposome suspension produced at 40 °C and at different pressures.

A FESEM image of liposome morphology produced at 150 bar, 40 °C is reported in **Figure 4**. Samples for microscopy were prepared using the procedure described in *Materials and Methods* section. Liposomes are approximatively spherical, with a wrinkled surface.

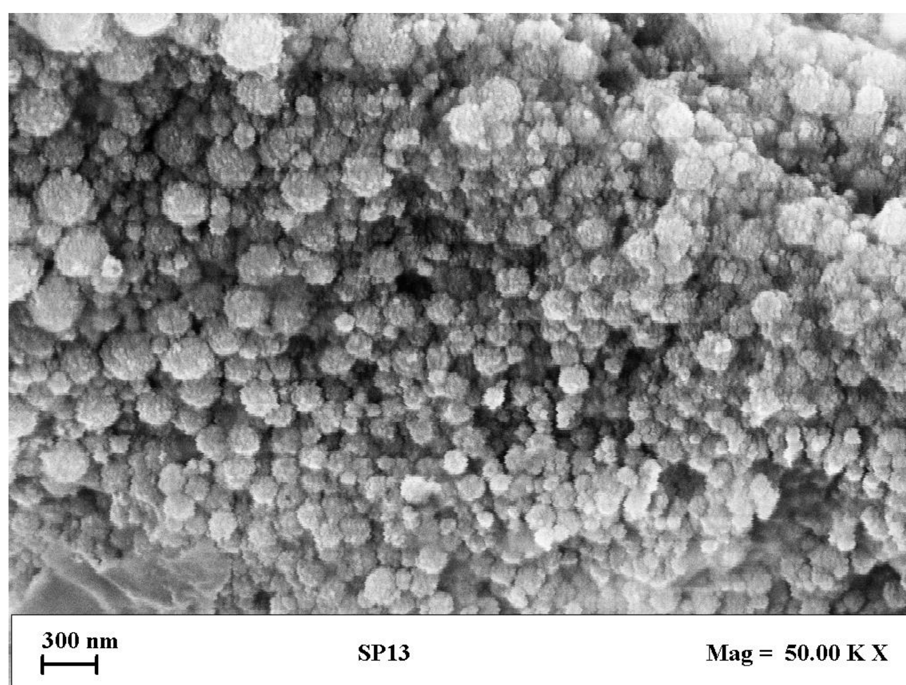


Figure 4 FESEM image of PC liposomes produced at 150 bar and 40 °C.

Zeta potential is a measure of the surface electrical charge of particles. The magnitude of the zeta potential gives an indication of the stability of the colloidal system: as the zeta potential increases, also repulsion between particles increases, leading to a more stable colloidal dispersion. If particles in suspension have a large negative or positive zeta potential they tend to repel each other. SuperLip process allows the direct production of stable liposomes with high and negative surface charge, with a zeta potential of about -20 mV.

Experiments at two temperatures (40 °C and 70°C) and at different precipitation pressures were also performed using a mixture of PC and PG, to explore the possibility of combining different phospholipids. They were dissolved in ethanol in the weight ratio 9/1, maintaining the overall lipid concentration in ethanolic solution at 5 mg/mL. Results are summarized in the **Table 1**. Using the mixture of phospholipids, slightly larger liposomes were obtained, when compared to the ones obtained using PC only, at the same process conditions: indeed, PC/PG liposome mean diameter varied between 350 (±101) and 280 (±70) nm when the pressure was increased from 125 and 175 bar.

A comparison of the results obtained at the same process conditions using PC and PC/PG mixture is plotted in **Figure 5**. It shows that, when PG is added to the formulation, lipid bilayer vesicles with larger dimensions are systematically obtained. This effect can be explained considering the higher steric volume of the PG polar head. The presence of PG in the lipid bilayer also produces an increase of the surface charge of liposomes: liposome suspensions prepared using PC/PG lipids were characterized by a higher negative zeta potential value (about -30 mV).

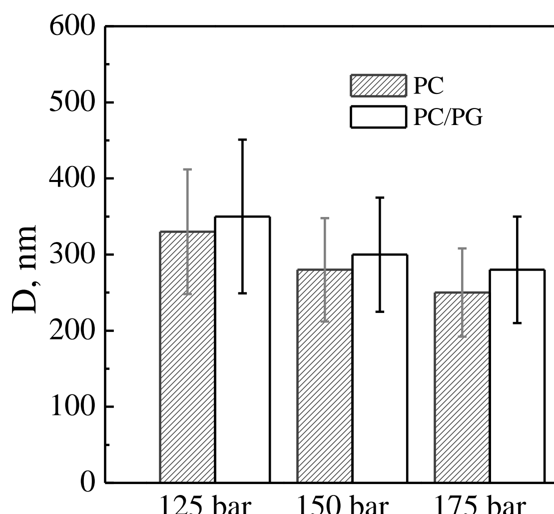


Figure 5 Liposome mean diameters obtained operating at different pressures and lipids composition

These experiments confirm the effect of higher process pressures in the reduction of suspensions PDI (see **Table 1,**).

Experiments using the PC/PG lipid mixture were also performed at a higher temperature (70 °C). Unimodal PSDs were obtained also in this case. A comparison of PSDs obtained at 40 °C and 70 °C for 125, 150 and 175 bar, is proposed in **Figure 6**. Increasing temperature, larger liposomes are produced (see **Table 1**), probably as the consequence of a reduction of expanded liquid density. Indeed, operating at 40 °C and for pressures between 125 and 175 bar expanded liquid densities between about 730-815 $Kg \cdot m^{-3}$ are obtained. For the same set of operative pressures, the fluid density was in the range 375-600 $Kg \cdot m^{-3}$ at 70 °C. This fact may have a role in reducing the atomization efficiency of water, thus producing larger droplets that could be later covered by the phospholipids producing larger liposomes.

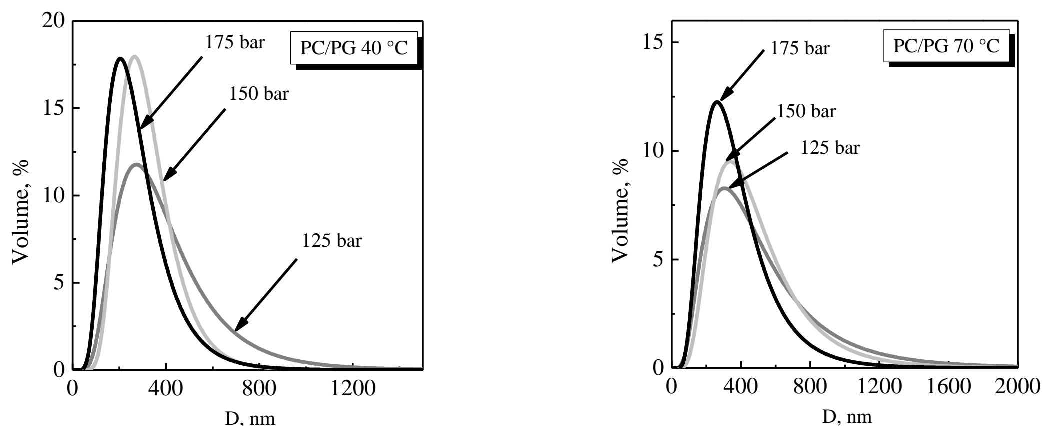


Figure 6 PSDs of PC/PG liposome suspensions obtained at different pressures at 40°C and 70°C.

Comparing the results obtained at different pressures and at different process temperatures (see **Figure 7)** a trend is evidenced: the higher the pressure, the lower the temperature (i.e. the higher the expanded liquid density) the smaller is the mean diameter of liposomes.

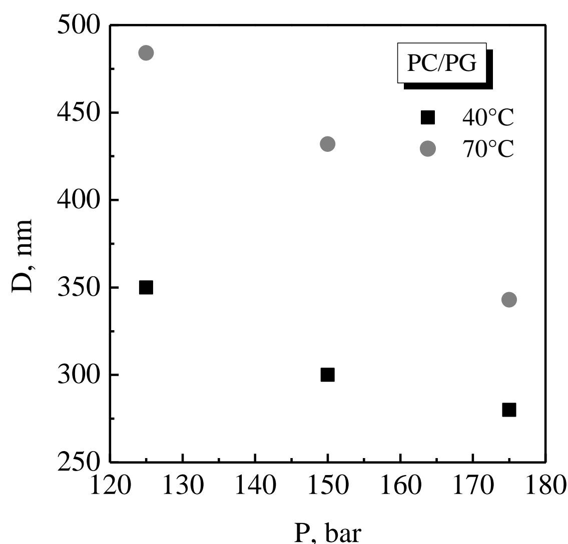


Figure 7 Effect of pressure and temperature on PC/PG liposome diameter at two different temperatures.

3.2 Production of loaded liposomes

To verify the encapsulation efficiency of hydrosoluble drugs using *SuperLip*, experiments were performed loading BSA in the water phase to be atomized and the encapsulation efficiency (EE) of the PC liposomes was measured. BSA was used as a model therapeutic protein. Loaded liposomes were produced at 150 bar, 40°C. GLR and CO_2 flow rate, ethanol flow rate and phospholipids concentration were maintained constant as in the previous experiments.

Liposomes with different BSA theoretical loading (10, 30 and 60%) were produced, as shown in **Table 2**. The theoretical loading is expressed as the percentage of BSA in the starting water solution with respect to the amount of phospholipids in the ethanol solution. The same

experiments were performed using the Bangham method, that consists of the hydration of a thin lipid film with a BSA solution, for comparison purposes.

Table 2. Encapsulation efficiency of BSA loaded liposomes obtained by SuperLip at 150 bar and 40 °C.

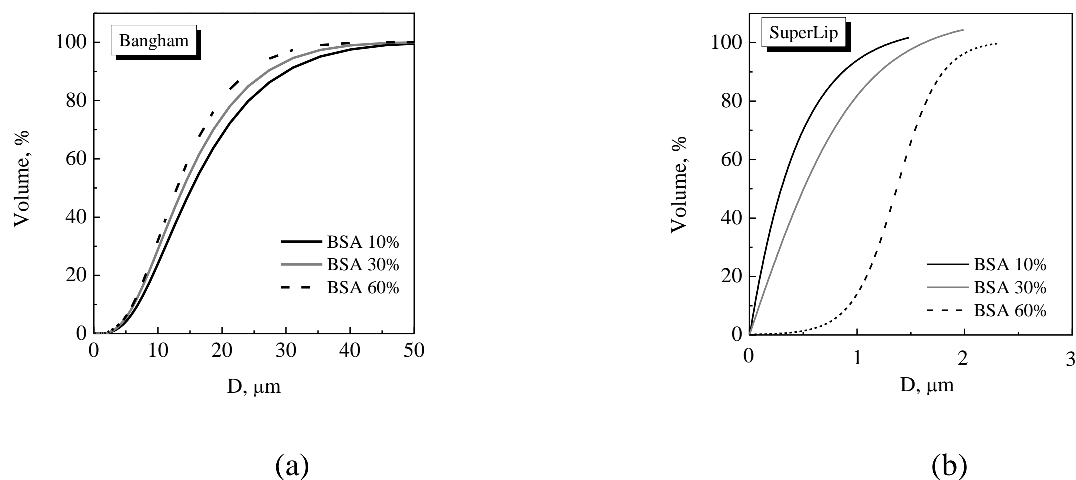
	Theoretical loading %	**Effective Loading**	**EE %**	**MD±SD μm**
Bangham	10	0.20	**2**	17.0±7.80
	30	6.60	**22**	15.3±6.90
	60	34.20	**57**	14.2±6.10
SuperLip	10	9.81	**98**	0.30 ± 0.07
	30	27.8	**92**	0.51 ± 0.11
	60	59.10	**98**	1.38 ± 0.30

Unilamellar micrometric liposomes were produced using the Bangham method, with an average diameter ranging between 14.2±6.1 and 17±7.8 μm.

PSD of liposomes produced using the Bangham method, at different concentration of BSA, is reported in Figure 9a. Large liposomes are produced and their mean diameter is practically not affected by the amount of BSA dissolved in the water solution used for hydration. Encapsulation efficiency of BSA was very low. As shown in Table 2, encapsulation efficiency was near to zero in the case of the lowest theoretical loading tested and was improved only increasing the theorical loading up to a final encapsulation efficiency of 57% in correspondence of the 60% theorical BSA loading with respect to phospholipids. In other worlds, it was found a large dependency of the encapsulation efficiency from the theoretical loading. This result can be explained considering the mechanism of liposome formation using the Bangham method. Indeed, the entrapment of molecules inside the bilayer vesicles, in this method, is a random phenomenon, that occurs during the hydration of the thin lipid film. When a more concentrated BSA solution is used for hydration there is an increased probability of entrapment of the desired active molecules inside the lipid vesicles, resulting in an improved encapsulation efficiency.

BSA loaded liposomes have been successfully produced using SuperLip. PSDs of loaded liposomes are reported in Figure 9(b). It can be observed that the diameter of produced liposomes strongly increased with the increase of the BSA concentration in water. Submicrometric PSDs have been obtained in the case of 10 and 30% of BSA loading; increasing the BSA loading to 60%, instead, micrometric PSD have been produced. The presence of the solute has a decisive influence on the PSDs of the suspensions. This result suggests that probably the dimensions of the liposomes are determined by the dimensions of the water droplets produced during atomization in the high pressure vessel. Indeed, increasing the solute concentration an increase in the solution density and viscosity is expected; i.e., the cohesive forces that obstacle the jet disruption increase, resulting in a reduction of atomization efficiency.

High encapsulation efficiencies were obtained for all the BSA loading tested, they ranged between 92-98%. This is a very important result that confirms the potential of SuperLip in the efficient production of therapeutic liposomes.



(a) (b)

Figure 9. BSA loaded liposomes produced using the Bangham method (a) and SuperLip (b)

The encapsulation efficiency was very similar for different BSA theoretical loading. This result is in agreement with the postulated mechanism of liposome formation during SuperLip: the formation of liposomes starts around the atomized water droplets, for this reason all the compounds that are dissolved in the water solution are automatically entrapped in the lipid layer, producing, as a result, a high encapsulation efficiency.

FTIR analysis of BSA loaded liposomes, compared to native BSA solution spectrum (Figure 10) shows that SuperLip did not damage the protein structure preserving its functionality. Indeed, from the deconvolution of FTIR spectrum it was observed that the α-helic content in native BSA is 66% and remained practically unchanged (at 64%) in processed BSA.

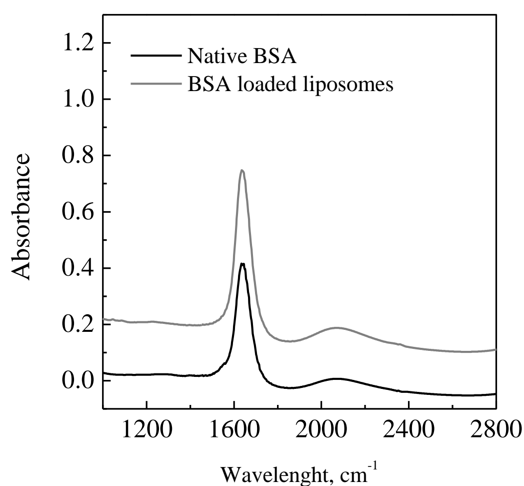


Figure 10. FTIR spectrum of native BSA solution and BSA loaded in to liposomes produced with SuperLip

4 CONCLUSIONS

The results produced in this work confirmed that the mechanism of liposome formation in SuperLip, is related to the favorable interactions between the expanded liquid phospholipid mixture and the atomized water droplets. This mechanism is also at the basis of the successful encapsulation of BSA into liposomes. Indeed, if aqueous droplets are rapidly surrounded by a layer of the lipids dissolved in the expanded liquid, these droplets, form a w/CO_2 emulsion and liposomes (w/w emulsion) are formed when fall in the water pool located at the bottom of the vessel. The process is very fast thanks to the large coefficients that characterize supercritical mixtures mass transfer. In this way, all the compounds dissolved in the water solution are efficiently entrapped inside the liposomes with a very high encapsulation efficiency, differently from the generality of the processes until now proposed. This result is the most relevant result of this work, that confirms the potential of SuperLip in producing, in an efficient and reliable manner, therapeutic liposomes. Furthermore, thanks to the possibility to operate at mild temperature conditions it is possible to encapsulate thermosensitive compounds, like proteins, preserving their functionality.

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