Preparation of liposomes encapsulating therapeutic proteins using 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 330 ± 82 nm and 250 ± 62 nm. Also phosphatidylglycerol (PG) had been used coupled with PC to produce liposomes. PC/PG larger liposomes have been produced in this case ranging between 350 ± 101 nm and 280 ± 95 nm. Drug encapsulation tests were also performed using fluorescein as a tracer compound and bovine serum albumin (BSA, used as a model therapeutic protein. Encapsulation tests have been performed with Fluorescein theoretical loading of 1, 3 and 6% and BSA theoretical loading of 10, 30, 60% with respect to the lipid amount. High encapsulation efficiencies (90-100%) were obtained for all the different drug loading tested, confirming that the active compound contained in the water phase can be efficiently entrapped in the formed vesicles using this new technique.

PC and PC/PG liposomes were very stable over time thanks to elevated and negative surface charge (zeta potential ranging between -20mV and -30 mV).

1 INTRODUCTION

Liposomes are vesicles in which 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 field, these carriers are being introduced with difficulties in the market and this is partly due to problems related with the 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 aqueous media [8-10]. These methods have some drawbacks, such as the large number of steps needed to produce the vesicles, the utilization of a large amount of organic solvent in the beginning or during the process, the lack of diameter size uniformity and, moreover, the low stability of produced liposomes [11].

In the field, of particle formation and carriers production, supercritical fluid technologies can be used to 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 [12-15]. Recently, some techniques based on the use of supercritical CO_2 ($scCO_2$) have been proposed also for liposomes preparation [16-21]; they try to take the advantage of the enhanced mass transfer of supercritical fluids [22] and can be divided in two categories: *two steps processes* in which the dried lipid particles need to be rehydrated [11, 20, 23-26], and *one step processes* in which a liposome-water suspension is directly obtained at the end of the process [27]. These kind of processes have still some limitation related to the control of liposomes dimension and distribution and also shows very low encapsulation efficiencies, especially for liposomes containing hydrophilic drugs (efficiencies around 10-20%) [28]. Only in case of certain protonatable amphiphilic water soluble drugs, liposomes can be sufficiently loaded thanks to favorable interaction of the drug and the lipidic membrane, but no general methods exist to encapsulate water soluble compounds without forming a conjugate sufficiently on a large scale into liposomes [29]. The limitation of conventional and supercritical fluids based processes is low encapsulation efficiency of hydrophilic drugs and is substantially due to the fact that in all these processes only a part of the water used to hydrate the lipids is effectively entrapped in the lipid membrane.

Reverchon and co-workers have recently proposed in the literature a new supercritical fluid based process for liposomes production [30]. The process is named Supercritical Assisted Liposome formation (SuperLip). Differently from the previously proposed techniques, the basic principle of this w process is to produce first water based micro and nanodroplets and, then, the liposomes are formed around them. Water solution droplets produced by atomization into an expanded liquid mixture formed by lipid compounds + ethanol + CO_2 were used. The 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 and a water in CO_2 emulsion is formed. At the end of this very fast 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 are obtained, that maintain a diameter similar to the original droplets produced during the atomization step. 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.

In this work the SuperLip process is applied to demonstrate the possibility to overcome the limitation of the conventional techniques regarding low encapsulation efficiency of hydrophilic drugs. First empty liposomes of soybean phosphatidylcholine (PC) and phosphatidylglycerol (PG) have been produced varying process operating parameters like pressure and temperature. Then, encapsulation tests were performed using fluorescein as tracer compound and bovine serum albumine (BSA) as therapeutic protein. Different drug loading have been tested to evaluate their effect on liposomes encapsulation efficiency.

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 throughout all the formulations. Trifluoroacetic acid (TFA 99%; Carlo Erba Reagents; Milan, Italy), Bovine serum albumin (BSA lyophilized powder ≥98%; Sigma-Aldrich; Milan, Italy) Fluorescein (Fluorescent tracer, Sigma-Aldrich; Milan, Italy). All the compounds were used as received.

2.2 SuperLip apparatus layout

Liposomes were prepared by the supercritical fluid assisted process called SuperLip. Briefly, at the beginning of the process, feeding pure CO_2, the operative pressure, temperature and gas mass flow conditions are reached in the saturator and the precipitation vessel; then a fixed volume (50 mL) of distilled water is delivered to the vessel to form a water volume at the bottom of the vessel in which the water droplets covered by the lipid layer are received, rearranging in the typical liposomes double layered structure. At the end of this preparatory procedure, the ethanolic phospholipidic solution is fed to the saturator together with high pressure CO_2. As a consequence, an expanded liquid mixture is formed in the saturator and then, is delivered to the precipitation vessel, where steady state compositions are obtained. A water solution (or distilled water in the experiments for empty liposomes formation) is atomized in the vessel using a 80 μm injector diameter and the droplets come in contact with the surrounding high pressure expanded liquid. Liposomes suspension is collected at fixed time intervals into a reservoir located downstream the high pressure vessel using an on-off valve.

A schematic representation of the apparatus is depicted in **Figure 1**. Detailed description of the apparatus is reported elsewhere[31].

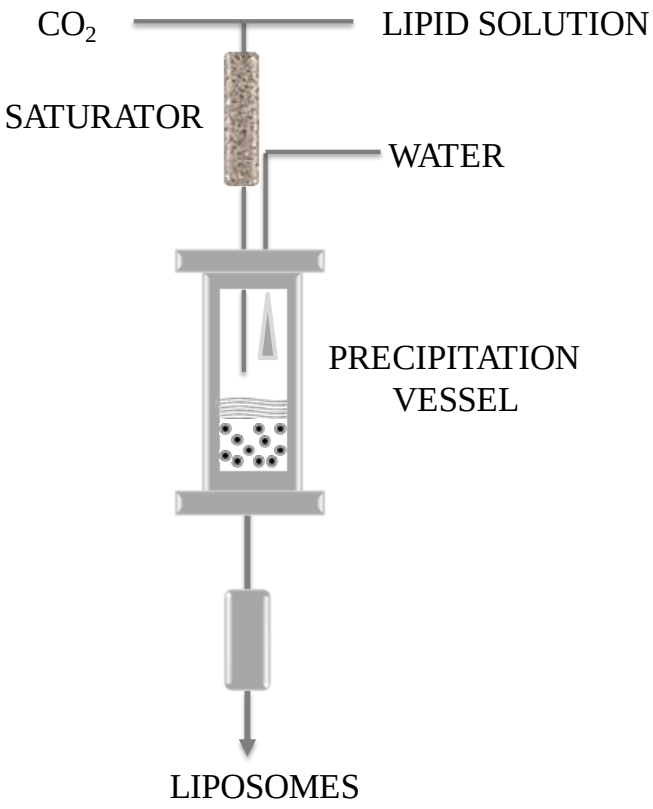

Figure 1: Schematic representation of the SuperLip process layout.

2.3 Liposomes characterization: morphology, size distribution and protein quantification

The morphology of the 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 for microscopy analysis using the following procedure. A drop of liposomes 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 (model B7341; Agar Scientific, Stansted, UK).

Particles size distribution (PSD), mean diameters (MD), polidispersity index (PDI) and zeta potential of the liposome suspensions were measured using a Malvern Zeta Sizer laser scattering instrument (mod. Zetasizer Nano S, Worcestershire, UK). 1 mL of the produced suspension was used for each test, without any further dilution step.

For the determination of encapsulation efficiencies, liposomes suspensions were centrifuged at 6500 for 45 minutes. After centrifugation the concentration of the drug in the supernatant (mg $_{drug}$) was analyzed using UV-vis spectroscopy, using a wave length of 280 nm for BSA and 515 nm for Fluorescein. The encapsulation efficiency was calculated with respect to the total theoretical drug content (mg $_{loaded}$) using the following equation:

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

The procedure of analyzing the supernatant concentration for the determination of encapsulation efficiency is discussed elsewhere [32].

3 RESULTS AND DISCUSSION

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

The first set of experiments was performed to form empty PC liposomes, and therefore, water only was atomized. Temperature inside the vessel and the mixer, was set at 40 °C and vessel and mixer pressure were set to 125, 150 or 175 bar. The nozzle used for atomization had 80 µm internal diameter and water flow rate was fixed at 10.0 mL/min. The gas/liquid ratio (GLR w/w) 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 the phospholipids ethanolic solution. PC concentration in ethanol solution was fixed at 0.2 mg/mL. Considering the high pressure phase equilibra data for the system CO_2-ethanol-water at 40 °C and in the 100-200 bar pressure range, (reported in the **Figure 2**), the selected process conditions ensure that the operative point, represented in the same figure, is located inside the miscibility hole where water and expanded liquid phase split in two phases and a water in the organic CO_2 solution (ethanol + PC) emulsion can be formed.

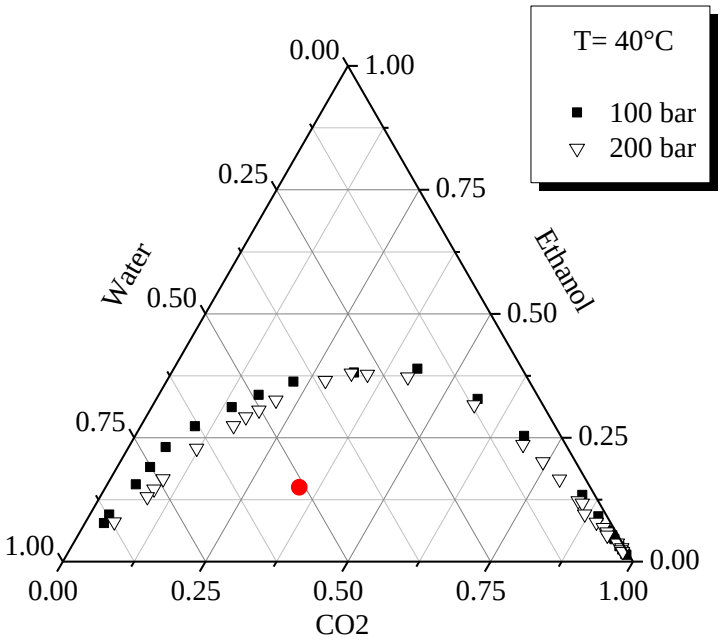

Figure 2. Phase equilibrium data for the CO_2-ethanol-water system at 40 °C and 100÷200 bar pressure range, adapted from [33]. The position of the operative point for the experiments performed at 125, 150 and 175 bar is also reported (red colored).

Table 1 reports the mean diameter of PC liposomes produced by SuperLip in these first experiments at different pressures. Liposomes mean diameter varied between 330 (±82) and 250 (±62) nm when the pressure was increased from 125

and 175 bar. Increasing the pressure also a sharping of the PSD amplitude was observed, lower PDI were obtained in correspondence of the higher value of pressure. A comparison of PSDs of liposome suspensions obtained at different pressures is reported also in **Figure 3**, where the effect of pressure on liposomes mean diameter and suspension PDI can be better appreciated. 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 [34] (pure CO_2 density data under pressure are reported in **Table 1**, calculated using the Bender equation of state [35]) that favors atomization of water injected in the high pressure vessel [36]. The generation of smaller water droplets, consequently, leads to the decrease of liposomes diameter.

Table 1. Liposomes size distributions obtained at 40 °C for different pressures. Process conditions: CO_2 flow rate 6.7 g/min, GLR= 2.42, water flow rate 10 mL/min, PC and PC/PG concentration in ethanol solution was fixed at 0.2 mg/mL, PC/PG ratio = 1/9, nozzle diameter 80 μm.

p(bar)	**CO_2 density (Kg m^{-3})**	**PC Mean size (nm ± SD)**	**PDI**	**PC/PG Mean size (nm ± SD)**	**PDI**
125	730	330±82	0.50	350±101	0.58
150	781	280±68	0.49	300±75	0.50
175	815	250±62	0.50	280±95	0.50

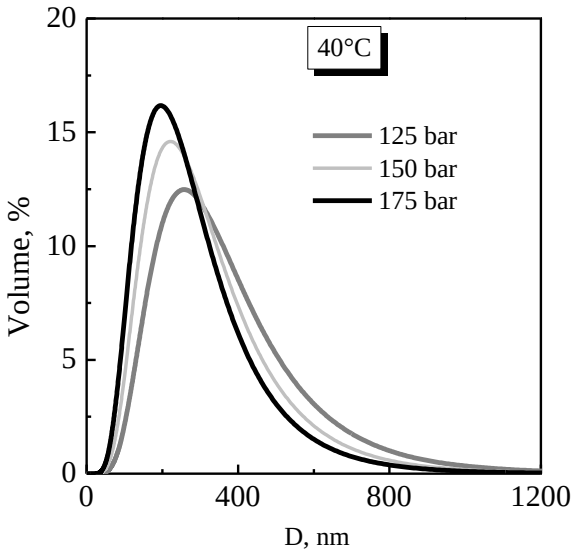

Figure 3. PSDs of PC liposomes suspension produced at 40 °C for different pressures.

A FESEM image of liposomes morphology obtained 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 arre approximatively spherical, with sub-micrometric dimensions and a rough surface.

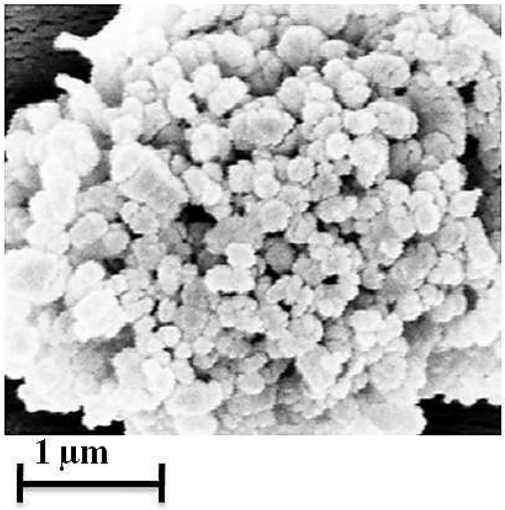

Figure 4 FESEM image of PC liposomes produced at 150 bar and 40 °C.

Zeta potential is a measure of the surface electrical charge of particles. The magnitude of the zeta potential gives an indication of the potential stability of the colloidal system. As the zeta potential increases, also repulsion between particles increases, leading to a more stable colloidal dispersion. If all particles in suspension have a large negative or positive zeta potential then they will tend to repel each other and there will be no tendency for the particles to stay together. 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 40 °C and at different precipitation pressures were also performed using a mixture of PC and PG in the weight ratio 9/1, maintaining the overall lipid concentration in ethanolic solution at 0.2 mg/mL. Results are reported in the **Table 1**.

Using a mixture of phospholipids, larger liposomes have been obtained, indeed PC/PG liposomes mean diameter varied between 350 (±101) and 280 (±95) nm when the pressure was increased from 125 and 175 bar, whereas PC liposomes mean diameter varied between 330 (±82) and 250 (±62) in the same process conditions.

A comparison of results obtained at the same process conditions using only PC and PC/PG mixture is plotted in **Figure 5**. This figure shows more evidently that, when PG is used in 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. PG in the lipid bilayer also produces a slight increase of the surface charge of liposomes: liposomes suspensions prepared using PC/PG lipids were characterized by a higher negative zeta potential value of about -30 mV.

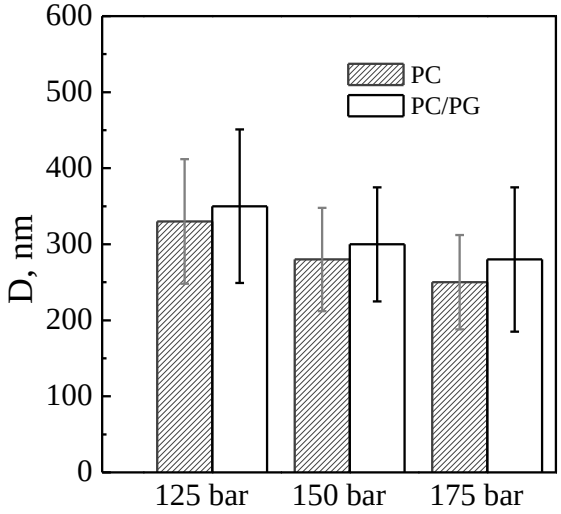

Figure 5 Liposomes mean diameters obtained at different pressure and lipids composition

Also in these experiments the effect of larger pressures is the reduction of liposomes dimensions and PDI (see **Table 1,**). These results confirm the trend observed in the case of PC liposomes, see also from **Figure 5.** Experiments using the PC/PG lipid mixture were also performed at a higher temperature (70 °C). Homogeneous 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 shown in **Figure 6**. Increasing temperature larger liposomes are produced as a consequence of a reduction of expanded liquid density. Indeed operating at 40 °C and for pressures between 125 and 175 bar expanded liquid densities of about 730-815 $Kg \cdot m^{-3}$ are obtained in the vessel, for the same set of operative pressures the fluid density decreased to 375-600 $Kg \cdot m^{-3}$ at 70 °C. It can have a determining role in reducing the atomization efficiency of water, thus producing larger droplets and then larger liposomes.

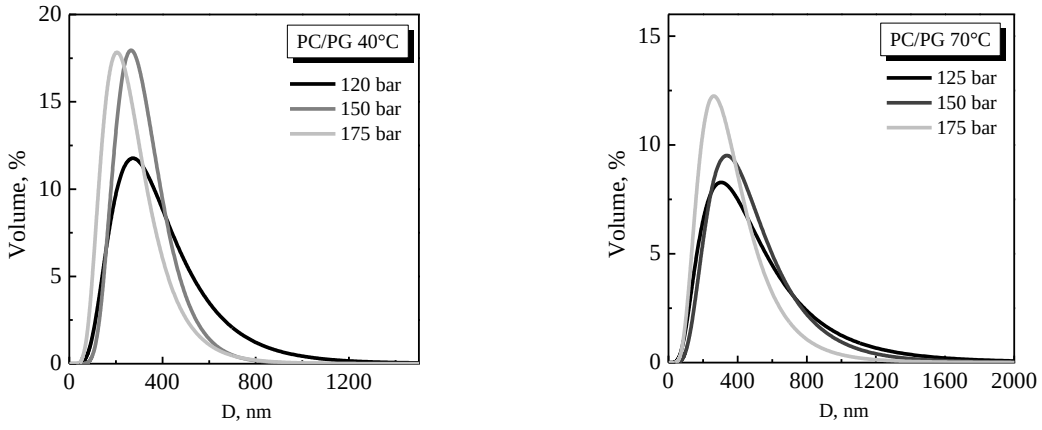

Figure 6 PSDs of PC/PG liposomes suspensions obtained at different pressures at 40°C and 70°C.

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

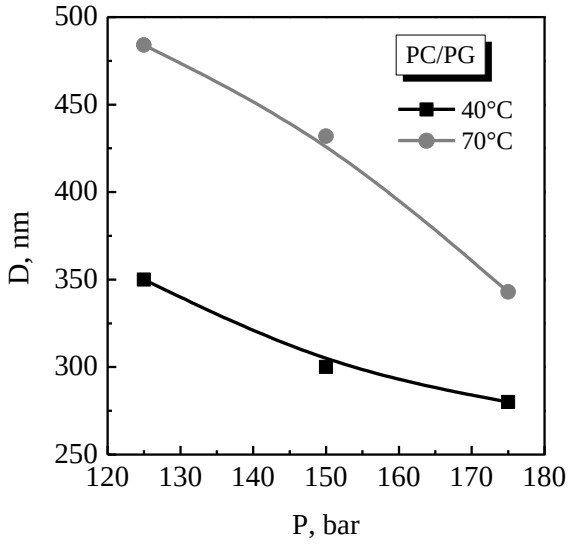

Figure 7 Effect of pressure on PC/PG liposomes diameter at two different temperatures.

3.2 Production of loaded liposomes

To demonstrate the potential improvement of encapsulation efficiency of hydrosoluble drugs using *SuperLip* process, first experiments were performed loading fluorescein in the water phase to be atomized and the encapsulation efficiency (EE) of the formed PC liposomes was measured. Fluorescein was used as a model hydrophilic compound. Loaded liposomes were produced at 150 bar, 40°C precipitation conditions. GLR and CO_2 flow rate, ethanol flow rate and phospholipids concentration were maintained constant as in the previous experiments. Liposomes with different fluorescein theoretical loading have been produced, as shown in **Table 2**. The theoretical loading is expressed as the percentage amount of fluorescein in the water solution with respect to the amount of phospholipids in the ethanol solution. Liposomes with an average diameters of about 220±72 nm have been obtained loaded with 1, 3 and 6% of fluorescein. Loaded and not loaded liposomes showed approximatively the same average diameter and particles size distribution. Elevated encapsulation efficiency was obtained for all the fluorescein loading tested, around 92-100%. Each experiment reported in Table 2 was repeated in triplicates; from the comparison of the results obtained from the encapsulation efficiency it appeared that the process is characterized by high reproducibility of the results.

The same experiments were performed using BSA as a model protein. Liposomes with the theoretical loading of 10, 30 and 60% have been produced in this case. PC liposomes encapsulating BSA have been successfully produced, with average diameters of 266±71 nm. Encapsulation efficiencies were elevated also for BSA between 93-98%.

Table 1. Encapsulation efficiency Fluorescein and BSA loaded liposomes obtained at 150 bar and 40 °C. Process conditions: CO_2 flow rate 6.7 g/min, GLR= 2.42, water flow rate 10 mL/min, PC and PC/PG concentration in ethanol solution was fixed at 0.2 mg/mL, nozzle diameter 80 µm.

	Theoretical loading %	EE	Real Loading
Fluorescein	1	**95.71**	0.96
	3	**99.65**	2.98
	6	**92.57**	5.55
BSA	10	**98.13**	9.81
	30	**92.74**	27.8
	60	**98.50**	59.10

Also PG/PC liposomes encapsulating BSA at 10% have been produced.

Liposomes with an average diameter of 350±90 nm were obtained. The encapsulation efficiency obtained during these tests ranged between 90 and 95% 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 the solute.

CONCLUSIONS

In conclusion in this work the SuperLip process has been applied efficiently to the production of loaded submicrometric liposomes obtaining high encapsulation efficiency (90-100%), with a good control of PSDs and reproducibility of results. These results are important, because confirm that the SuperLip process can overcome the common limitations of liposomes preparation techniques. To obtain this result it was necessary to change the principal steps involved in liposomes preparation processes. Usually all the techniques started with lipid layer or dry particles production and subsequently they are rehydrated with water; in this way encapsulation efficiency cannot be high even from a theoretical point of view. The

SuperLip process was developed focusing the attention of the water internal phase of liposomes; for this reason in this process the basic part is the atomization of water+solute solution in a high pressure vessel in which phospholipids are dissolved in an expanded liquid. Results obtained in this work, give a confirmation of the postulated mechanism of liposomes formation during the process. In particular, the efficiency of water atomization determines liposomes dimension because liposomes are formed around atomized water droplets maintaining the dimension of the generating droplet, thus ensuring a high encapsulation efficiency.

REFERENCES

[1] A.D. Bangham, M.W. Hill, N.G.A. Miller, Preparation and Use of Liposomes as Models of Biological Membranes, in: E. Korn (Ed.) Methods in Membrane Biology, Springer US, 1974, pp. 1-68.
[2] G. Storm, D.J.A. Crommelin, Liposomes: quo vadis?, Pharmaceutical Science & Technology Today, 1 (1998) 19-31.
[3] K.J. Harrington, K.N. Syrigos, R.G. Vile, Liposomally targeted cytotoxic drugs for the treatment of cancer, The Journal of pharmacy and pharmacology, 54 (2002) 1573-1600.
[4] M.A. Kisel, L.N. Kulik, I.S. Tsybovsky, A.P. Vlasov, M.S. Vorob'yov, E.A. Kholodova, Z.V. Zabarovskaya, Liposomes with phosphatidylethanol as a carrier for oral delivery of insulin: studies in the rat, International Journal of Pharmaceutics, 216 (2001) 105-114.
[5] F. Zho, M.R. Neutra, Antigen delivery to mucosa-associated lymphoid tissues using liposomes as a carrier, Bioscience reports, 22 (2002) 355-369.
[6] M.L. Matteucci, D.E. Thrall, The role of liposomes in drug delivery and diagnostic imaging: a review, Veterinary radiology & ultrasound : the official journal of the American College of Veterinary Radiology and the International Veterinary Radiology Association, 41 (2000) 100-107.
[7] M.C. Pedroso de Lima, S. Neves, A. Filipe, N. Duzgunes, S. Simoes, Cationic liposomes for gene delivery: from biophysics to biological applications, Current medicinal chemistry, 10 (2003) 1221-1231.
[8] S. Batzri, E.D. Korn, Single bilayer liposomes prepared without sonication, Biochimica et Biophysica Acta (BBA) - Biomembranes, 298 (1973) 1015-1019.
[9] O.R. Justo, Â.M. Moraes, Analysis of process parameters on the characteristics of liposomes prepared by ethanol injection with a view to process scale-up: Effect of temperature and batch volume, Chemical Engineering Research and Design, 89 (2011) 785-792.
[10] T. Wang, Y. Deng, Y. Geng, Z. Gao, J. Zou, Z. Wang, Preparation of submicron unilamellar liposomes by freeze-drying double emulsions, Biochimica et biophysica acta, 1758 (2006) 222-231.
[11] L. Lesoin, C. Crampon, O. Boutin, E. Badens, Preparation of liposomes using the supercritical anti-solvent (SAS) process and comparison with a conventional method, Journal of Supercritical Fluids, 57 (2011) 162-174.
[12] R. Campardelli, M. Cherain, C. Perfetti, C. Iorio, M. Scognamiglio, E. Reverchon, G. Della Porta, Lipid nanoparticles production by supercritical fluid assisted emulsion–diffusion, The Journal of Supercritical Fluids, 82 (2013) 34-40.
[13] I. De Marco, M. Rossmann, V. Prosapio, E. Reverchon, A. Braeuer, Control of particle size, at micrometric and nanometric range, using supercritical antisolvent precipitation from solvent mixtures: application to PVP, Chemical Engineering Journal.
[14] Š. Petra, A. Renata, K. Věra, R. Ernesto, S. Tomáš, P. Miroslav, Supercritical assisted atomization of emulsions for encapsulation of 1-monoacylglycerols in an hydrophilic carrier, Journal of Supercritical Fluids, 97 (2015) 183-191.
[15] P. Pisanti, A.B. Yeatts, S. Cardea, J.P. Fisher, E. Reverchon, Tubular perfusion system culture of human mesenchymal stem cells on poly- L- Lactic acid scaffolds produced using a supercritical carbon dioxide-assisted process, Journal of Biomedical Materials Research - Part A, 100 A (2012) 2563-2572.

[16] L. Lesoin, O. Boutin, C. Crampon, E. Badens, CO_2/water/surfactant ternary systems and liposome formation using supercritical CO_2: a review, Colloids and Surfaces A: Physicochemical and Engineering Aspects, 377 (2011) 1-14.
[17] L.A. Meure, N.R. Foster, F. Dehghani, Conventional and dense gas techniques for the production of liposomes: a review, AAPS PharmSciTech, 9 (2008) 798-809.
[18] L. Frederiksen, K. Anton, P.v. Hoogevest, H. Keller, H. Leuenberger, Preparation of liposomes encapsulatins water-soluble compounds using supercritical carbon dioxide, Journal of Pharmaceutical Sciences, 86 (1997) 921-928.
[19] K. Otake, T. Imura, H. Sakai, M. Abe, Development of a new preparation method of liposomes using supercritical carbon dioxide, Langmuir, 17 (2001) 3898-3901.
[20] E. Badens, C. Magnan, G. Charbit, Microparticles of soy lecithin formed by supercritical processes, Biotechnology and Bioengineering, 72 (2001) 194-204.
[21] M. Cano-Sarabia, N. Ventosa, S. Sala, C. Patiño, R. Arranz, J. Veciana, Preparation of uniform rich cholesterol unilamellar nanovesicles using CO_2-expanded solvents, Langmuir, 24 (2008) 2433-2437.
[22] L.A. Meure, R. Knott, N.R. Foster, F. Dehgani, The depressurizationof an expanded solution into aqueous media for the bulk production of liposomes, Langmuir, 25 (2009) 326-337.
[23] F. Xia, H. Jin, Y. Zhao, X. Guo, Supercritical antisolvent-based technology for preparation of vitamin D_3 proliposome and its characteristics, Chinese Journal of Chemical Engineering, 19 (2011) 1039-1046.
[24] S. Kunastitchai, L. Pichert, N. Sarisuta, B.W. Müller, Application of aerosol solvent extraction system (ASES) process for preparation of liposomes in a dry and reconstitutable form, International Journal of Pharmaceutics, 316 (2006) 93-101.
[25] U.S. Kadimi, D.R. Balasubramanian, U.R. Ganni, M. Balaraman, V. Govindaraiulu, In vitro studies on liposomal amphotericin B obtained by supercritical carbon dioxide-mediated process, Nanomedicine: Nanotechnology, Biology and Medicine, 3 (2007) 273-280.
[26] Y. Li, D.-J. Yang, S.-L. Chen, S.-B. Chen, A.S.-C. Chan, Process parameters and morphology in puerarin, phospholipids and their complex microparticles generation by supercritical antisolvent precipitation, International Journal of Pharmaceutics, 359 (2008) 35-45.
[27] T.P. Castor, L. Chu, Methods and apparatus for making liposomes containing hydrophobic drugs, in, 7 lug 1998, US5776486 A.
[28] L. Lesoin, C. Crampon, O. Boutin, E. Badens, Development of a continuous dense gas process for the production of liposomes, Journal of Supercritical Fluids, 60 (2011) 51-62.
[29] I. Cabrera, E. Elizondo, O. Esteban, J.L. Corchero, M. Melgarejo, D. Pulido, A. Córdoba, E. Moreno, U. Unzueta, E. Vazquez, I. Abasolo, S. Schwartz, A. Villaverde, F. Albericio, M. Royo, M.F. García-Parajo, N. Ventosa, J. Veciana, Multifunctional Nanovesicle-Bioactive Conjugates Prepared by a One-Step Scalable Method Using CO2-Expanded Solvents, Nano Letters, 13 (2013) 3766-3774.
[30] I.E. Santo, R. Campardelli, E.C. Albuquerque, S.V. de Melo, G. Della Porta, E. Reverchon, Liposomes preparation using a supercritical fluid assisted continuous process, Chemical Engineering Journal, 249 (2014) 153-159.
[31] I. Espirito Santo, R. Campardelli, E. Cabral Albuquerque, S. Vieira de Melo, G. Della Porta, E. Reverchon, Liposomes preparation using a supercritical fluid assisted continuous process, Chemical Engineering Journal, 249 (2014) 153-159.

[32] T. Nii, F. Ishii, Encapsulation efficiency of water-soluble and insoluble drugs in liposomes prepared by the microencapsulation vesicle method, International Journal of Pharmaceutics, 298 (2005) 198-205.
[33] N.E. Durling, O.J. Catchpole, S.J. Tallon, J.B. Grey, Measurement and modelling of the ternary phase equilibria for high pressure carbon dioxide-ethanol-water mixtures, Fluid Phase Equilibr, 252 (2007) 103-113.
[34] H. Pöhler, E. Kiran, Volumetric Properties of Carbon Dioxide + Ethanol at High Pressures, Journal of Chemical & Engineering Data, 42 (1997) 384-388.
[35] B. Platzer, G. Maurer, A generalized equation of state for pure polar and nonpolar fluids, Fluid Phase Equilibria, 51 (1989) 223-236.
[36] G. Caputo, R. Adami, E. Reverchon, Analysis of Dissolved-Gas Atomization: Supercritical CO2 Dissolved in Water, Ind Eng Chem Res, 49 (2010) 9454-9461.