



Preparation of liposomes using the supercritical anti-solvent (SAS) process and comparison with a conventional method

L. Lesoin, C. Crampon, O. Boutin, E. Badens^{*,1}

Université Paul Cézanne Aix-Marseille, Laboratoire de Modélisation, Mécanique et Procédés Propres (UMR CNRS 6181), BP 80, 13 545 Aix-en-Provence cedex 4, France

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ABSTRACT

Two methods to produce liposomes encapsulating a fluorescent marker were compared: the supercritical anti-solvent (SAS) method and a conventional one (Bangham). Liposome size and encapsulation efficiency were measured to assess the methods. Micronized lecithin produced by the SAS process was characterized in terms of particle size, morphology and residual solvent content in order to investigate the influence of experimental parameters (pressure, CO₂/solvent molar ratio and solute concentration). It appears that when the lecithin concentration increases from 15 to 25 wt.%, at 9 MPa and 308 K, larger (20–60 μm) and less aggregated lecithin particles are formed. As concerns liposomes formed from SAS processed lecithin, size distribution curves are mainly bimodal, spreading in the range of 0.1–100 μm. Liposome encapsulation efficiencies are including between 10 and 20%. As concerns the Bangham method, more dispersed liposomes were formed; encapsulation efficiencies were about 20%, and problems of reproducibility have been raised.

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1. Introduction

Finely divided particles of phospholipids are of great interest to form controlled drug delivery systems (DDS) called liposomes. Phospholipids are natural surfactants and in the presence of water, they organize themselves in order to reduce the unfavourable interactions between their hydrophobic tails and the aqueous solution; their hydrophilic head groups expose to the aqueous phase forming vesicles (Fig. 1). Liposomes are featured by clearly separated hydrophilic and hydrophobic regions. A special characteristic of liposomes for drug delivery is that water-soluble and water-insoluble materials can be encapsulated together. Water-soluble materials are entrapped in the aqueous core, while water-insoluble drugs reside within the phospholipid bilayer [1].

Over the last 30 years, the use of liposomes as DDS has expanded considerably [2]. Because they are non-toxic, biodegradable and non immunogenic, liposomes serve as convenient delivery vehicles for biologically active compounds [3]. Liposomes have been widely used in cancer treatment. For example, specific agents entrapped in liposomal carriers can be targeted to an organ and distinctions can be made between normal and tumorous tissues using computed tomography [4–7]. Moreover, the main benefit of using liposomes to deliver anti-cancer agents is that liposomes enable (i) to reduce

the toxic effects of the drugs and (ii) to increase the circulation time and effectiveness of the drugs [8–12]. Concerning the last point, liposomes as DDS enable once-daily medication which is a major breakthrough for patient everyday life. Liposomes are also used for gene therapy, as DNA delivery vectors which turns out to be also one of the main hopes of liposome medical use [13–20]. Lastly, DNA vaccination with liposomes is under way [21,22].

For drug delivery, liposomes can be formulated as a suspension, as an aerosol or in a (semi)solid form such as a gel, cream or dry powder; in vivo, they can be administered topically [23–26] or parenterally [3]. This study focuses on the formation of liposomes both in solid and suspension form. A wide variety of conventional methods exists to produce liposomes [27], including the Bangham method or thin layer evaporation method [28], the reverse phase evaporation method [29], the detergent depletion method [30,31], the ether/ethyl alcohol injection method [29,32] and the emulsion method [31]. These methods suffer from lots of drawbacks and especially the use of organic solvents with high-level of toxicity such as isopropyl ether, diethyl ether, chloroform and methyl alcohol [33].

Given the widespread interest in the use of liposomes in pharmaceuticals and cosmetics, development of new processes in compliance with the constraints imposed by Good Manufacturing Practises (GMP) is required. The nature (toxicity) of the organic solvents involved in the process and the residual organic solvent level in the final material are as important as liposome characteristics (size, encapsulation efficiency and stability) to assess the process.

^{*} Corresponding author.

E-mail address: elisabeth.badens@univ-cezanne.fr (E. Badens).

¹ Tel.: +33 442 90 85 00; fax: +33 442 90 85 15.

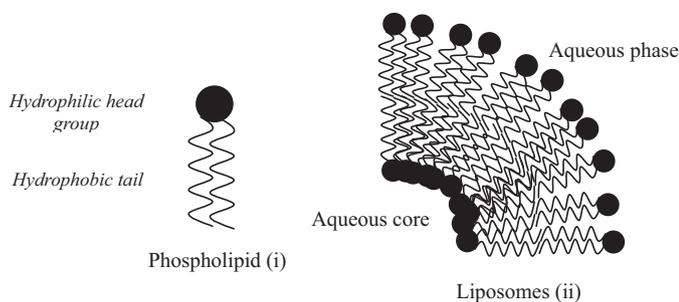


Fig. 1. Schematic representation of a phospholipid (i) and liposomes (ii).

To cope with these issues, dense gas techniques and especially supercritical carbon dioxide (CO₂) techniques [33–50] have been developed. With these techniques, the use of organic solvents is not always avoided but when they are needed, they have lower toxic level than the organic solvents used in conventional methods.

This study focuses on the application of the well known supercritical anti-solvent (SAS) method to comminute raw lecithin. In a second step, the hydration of the processed material is carried out to form liposomes. The SAS process has often been used for the precipitation of compounds difficult to comminute. The principle is to put into contact an organic solution containing the solute to be precipitated with a supercritical fluid, such as supercritical CO₂. This latter acts as an anti-solvent for the solute but is completely miscible with the organic solvent. The last point deserves to be highlighted because in the SAS process, the final processed material is deprived of any traces of organic solvents. Previous works have already described the micronization of phospholipids by SAS process [33,51–54]. The study presented here is more complete since the formation of the liposomes after the micronization has been systematically carried out and the characteristics of resultant liposomes have been discussed. Thus, influence of experimental parameters of the SAS process (pressure, CO₂/solvent molar ratio, solute concentration) on processed phospholipids and final liposomes characteristics is assessed.

Finally, the conventional Bangham method is also implemented in this study. SAS and Bangham processes are compared according to the residual solvent content of processed lecithin, size, encapsulation efficiency and stability of liposomes.

2. Materials and methods

2.1. Chemicals

Soy lecithin S75 (71% phosphatidylcholine) was used as phospholipids and purchased from LIPOID (Ludwigshafen, Germany). Analytical grade analysis ethyl alcohol was obtained from Sigma Aldrich (St Louis, MO). Instrument grade carbon dioxide (purity of 99.7%) from Air Liquide Méditerranée (Vitrolles, France) was used. Cholesterol, 4',5'-bis[N,N-bis(carboxymethyl)aminomethyl]fluorescein (calcein), Cobalt(II) chloride hexahydrate and non-ionic surfactant Triton X-100 (octylphenol polyethoxylated) were purchased from Sigma Aldrich (St Louis, MO) and used to assess the encapsulation efficiency of liposomes. Double distilled and deionized water was used throughout the experiments.

2.2. Preparation of liposomes

Our study is focused on the semi-continuous supercritical anti-solvent precipitation process, called SAS. Preparing liposomes via the SAS process has to be performed in two steps. Firstly, divided phospholipid microparticles are produced from raw soy lecithin through the SAS process. Secondly, microparticles are hydrated at ambient conditions by an aqueous solution of calcein (encapsulated marker) under stirring. As a reference control for our experiments, the conventional Bangham method [28,31,55], described later, is also used to prepare liposomes.

2.2.1. Phospholipid microparticle production

2.2.1.1. Experimental set-up for the SAS process. The experimental set-up for SAS process was described elsewhere [33,53] and is illustrated in Fig. 2. Briefly, the SAS process involved the co-currently spraying of a solution composed of the dissolved solute (soy lecithin) and of the organic solvent (absolute ethyl alcohol) through a capillary tube (127 μm I.D.; Chrompack, Les Ulis, France) into a continuous supercritical phase. The supercritical fluid used is supercritical carbon dioxide (CO₂). The simultaneous dissolution of the supercritical fluid in the liquid phase and the evaporation of the organic solvent in the supercritical phase lead to the supersaturation of the solute into the liquid phase and then its precipitation. For all experiments, the solution was sprayed in the precipitation vessel (stainless steel high pressure vessel, 650 cm³) once the

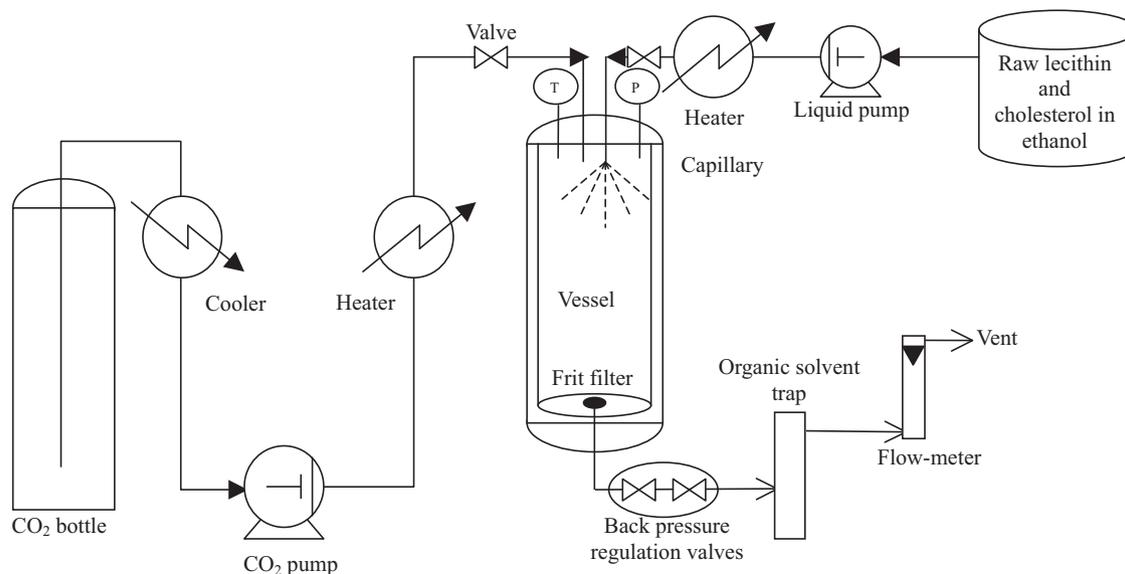


Fig. 2. Experimental set-up for the SAS process.

steady state (constant CO₂/solvent molar ratio in the vessel) was reached [56,57]. All experiments were carried out at 308 K, with a liquid flow rate of 22.8 mL h⁻¹ and ethyl alcohol as organic solvent. Moreover, for each experiment, 2 g of lecithin were precipitated in the high pressure vessel. Gas CO₂ was transformed into liquid CO₂ with a cooler and pumped with a liquid high pressure pump (Dosapro Milton Roy, Pont-Saint-Pierre, France). Then, liquid CO₂ was converted into gas CO₂ with a heater at the exit of the pump. The organic solution was injected in the precipitation vessel with a high pressure liquid pump Gilson 307 (Villiers le Bel, France). The bottom of the precipitator vessel was equipped with a frit filter. After the injection phase, a washing step was carried out in order to completely remove ethyl alcohol (collected in the solvent trap). The washing step was performed with pure CO₂ in order to renew the content of the autoclave with the same operating conditions as previously. Given that the CO₂ phase is lighter than the CO₂ + ethyl alcohol phase, the autoclave behaves as a plug flow reactor [56,57]. Thus, the time needed to renew the content of the autoclave was calculated. At the end of the process, after depressurization, small samples of microparticles were collected and immediately submitted to characterization or hydration to form liposomes.

2.2.1.2. Experimental set-up for the Bangham process. Conventional Bangham method was implemented to process raw lecithin. The Bangham method is divided in two steps: formation of a thin lipid film and mixing with an excess aqueous phase, i.e. hydration. The second step is presented in the next section. To form the lipid film, lecithin was dissolved in absolute ethyl alcohol. Then, the solvent was removed by evaporation (323 K) with a rotary evaporator 4000eco (Heidolph Laborota). After a while, an homogeneous film of phospholipids was formed at the surface of the evaporator flask.

2.2.2. Hydration step and encapsulation

It has to be noted that the micronized phospholipid particles (SAS process) and the dry phospholipid film (Bangham process) were hydrated in the same way to produce liposomes encapsulating calcein (fluorescent marker). Calcein solution (0.062 g mL⁻¹) was added in the vessel or the evaporator flask once the SAS process or the Bangham process has been performed. Then, stirring was realized using a high speed mixer Ultraturax T25 (Ika Labor Technik, Staufen, Germany) at 11 000 tr min⁻¹ during 10 min. According to Oku et al. [58], the volume of calcein solution was determined given that the weight ratio calcein/lecithin should be equal to 0.008. All liposomal suspensions were stored at 277.5 K.

2.3. Characterization

2.3.1. Phospholipid microparticles

2.3.1.1. Yield. Micronization yield was calculated as the ratio between lecithin recovered in the vessel and lecithin effectively introduced in the vessel during the SAS process.

2.3.1.2. Particle size and morphology. The particle size and morphology of the processed and unprocessed materials were observed using a Hitachi S-3000 (Hitachi, Japan) Scanning Electron Microscope (SEM). Each sample was prepared with a SC7620 Sputter Coater (Quorum Technologies, England) which deposited a 2 nm layer of gold and palladium in order to optimize the image resolution. Only qualitative observations of images were performed to assess size and shape of the particles. Statistical analysis was not undertaken.

2.3.1.3. Control of residual solvent content. A comparative method was implemented to assess the content of residual solvent in processed phospholipids. Thus, phospholipid microparticles produced

by the SAS method were compared to unprocessed phospholipid samples which were previously loaded with known amount of absolute ethyl alcohol. InfraRed Spectroscopy was used to compare the samples. Two characteristics absorption bands were chosen at 1735 cm⁻¹ and 1058 cm⁻¹ to assess the presence of solvent. The first one is representative of the carboxyl group of the lecithin; the second one features the alcohol group, i.e. ethyl alcohol. For each sample, the height ratio of the absorption bands $h_{1058\text{ cm}^{-1}}/h_{1735\text{ cm}^{-1}}$ was calculated. This ratio was taken into account to assess the content of residual solvent.

2.3.2. Hydrated particles or liposomes

2.3.2.1. Microscopic observations of liposomes. Liposomes were observed with a phase contrast optical microscope Reichert-Jung, Polyvar (plan objective 4×/10×/25×/40×) to characterize their morphology, shape and size.

2.3.2.2. Liposome size distribution and stability. Particle size distribution was measured by laser diffraction using Malvern Mastersizer S (laser 663 nm) (Malvern Instrument S.A., Orsay, France). The instrument covers a particle size range between 50 nm and 3 mm. All measurements were carried out triplicate. Size distribution curves show liposome population in volume percentage according to liposome size in micrometer. Size distributions are analysed according to the calculation of liposome cumulative volume percent for diameters below 1 μm, between 1 and 10 μm and above 10 μm. In order to study liposome stability, size measurements were carried out at $t=0$ and at $t=1$ month ($t=0$ stands for the day of production).

2.3.2.3. Encapsulation efficiency. (For encapsulation efficiency assessments, cholesterol was added in the organic solution – cholesterol/lecithin weight ratio=0.49 – before processing to enhance liposome stability in suspension form [59,60]).

Calcein (fluorescent marker) was encapsulated during the hydration step (Section 2.2.2). After this stage, a fraction of calcein is encapsulated in the aqueous core of liposomes and the rest fraction or the non-encapsulated part is in the aqueous solution. The encapsulation efficiency is defined as the percent of calcein encapsulated in liposomes. The method implemented in this study uses the ability of non-permeant Co²⁺ to completely quench the fluorescence of calcein through the formation of a Co-calcein complex, as described by Kendall and MacDonald [61]. Thus, Co²⁺ is used to complex the non-encapsulated calcein, i.e. to quench the fluorescence of the non-encapsulated calcein. The encapsulated fraction of calcein is protected from Co²⁺ by liposome bilayer. The procedure was first experimented by Oku et al. [58]. Briefly, the liposomal suspension was first diluted 200-fold in distilled water, and fluorescence intensity was measured before (F_{Total}) and after the addition of CoCl₂ ($F_{\text{Encapsulated}}$) and Triton X-100 (F_{Totalq}). As Co²⁺ is non-permeant, it forms a complex only with non-entrapped calcein and $F_{\text{Encapsulated}}$ refers to the fluorescence contribution of encapsulated calcein. Triton X-100 is a non-ionic detergent. Interactions between Triton X-100 and liposomal membranes have been clearly established by Hertz and Barenholz [62]. First of all, they found that the effect of Triton X-100 is time dependant. Then, they showed that when treated with Triton X-100, most of the lipid bilayers were damaged and seemed to have fused. The product of this fusion was a complex network of lipid layers. Concerning the release of encapsulated marker (glucose), Hertz and Barenholz [62] noticed that several milligrams of Triton X-100 yield to the release of 90% of an encapsulated marker. F_{Total} typically represented less than 5% of corresponding F_{Total} [63]. The encapsulation efficiency was then worked out as followed [58]: $EE\% = (F_{\text{Encapsulated}} - F_{\text{Totalq}})/(F_{\text{Total}} - F_{\text{Totalq}}) \times 100$. Fluorescent measurements were carried out with a Perkin Elmer

Luminescence spectrometer LS50 (Perkin Elmer, Courtaboeuf, France). Synchronous excitation emission mode was used from 400 to 600 nm (excitation slit: 5; emission slit: 10; wavelength interval: 23). All measurements were carried out triplicate. From the procedure described by Oku et al. [58], several fits need to be done. First of all, a 80 mL flask of diluted liposomal suspension was used. Samples for analysis were taken from this flask. After the determination of F_{Total} , 200 μL of CoCl_2 (10 mM) was added and the mixture was stirred with a glass mixer. Time for reaction between calcein and Co^{2+} was estimated at 15 min. Thus, measurements of $F_{\text{Encapsulated}}$ were performed 15 min after introducing the CoCl_2 solution. Lastly, approximately 3 mL of Triton X-100 was introduced in the flask and vigorous stirring (with a glass mixer) was needed to solubilize Triton X-100. Investigations showed that measurement of F_{Total} needs to be done after 48 h.

3. Results

3.1. SAS process

SAS experiments were conducted in the following experimental domain:

- Precipitation temperature: 308 K.
- Range of precipitation pressure: from 9 to 13 MPa.
- Lecithin concentration range: from 15 to 25 wt.%.
- Solution (raw lecithin and cholesterol in ethyl alcohol) flow rate: 22.8 mL h^{-1} .
- $\text{CO}_2/\text{solvent}$ (ethyl alcohol) molar ratio range: from 50 to 100.

Experimental values are summarized in Table 1. $\text{CO}_2/\text{lecithin}$ weight ratio is afforded in Table 1 in view of industrial scale-up [64].

Pressure, lecithin concentration and $\text{CO}_2/\text{solvent}$ molar ratio were the three SAS process variables whose influence was studied upon:

- Yield, particle size, particle morphology and residual solvent level for dry phospholipid powders.
- Size distribution and encapsulation efficiency of liposomes.

The reproducibility of the experimental results had been checked.

3.1.1. Yield

The SEM images demonstrate that agglomerated spherical microparticles of lecithin were obtained from SAS process (Figs. 4, 6 and 9). The micronized lecithin was light yellow-white and localised in the bottom of the vessel, as shown in Fig. 3. The production yields were between 75 and 85%. The explanation for the rather low yield obtained is that losses of precipitated particles would inevitably occur. Firstly, phospholipids are slightly soluble in the supercritical phase which contains ethyl alcohol. Consequently,



Fig. 3. Image of micronized lecithin at the bottom of the vessel.

some precipitated particles of phospholipids are carried along with the CO_2 flow and, as a proof some particles were recovered in the solvent trap. Summing the weight recovered in the autoclave and the weight found in the solvent trap, the weight balance was not checked. One may assume that given the difficulty to collect precipitated particles in the inside wall of the autoclave, some precipitated material may remain in the vessel. Such losses would be reduced using a larger vessel and bigger quantities of lecithin.

3.1.2. Effect of experimental parameters

3.1.2.1. Influence of precipitation pressure. Influence of precipitation pressure on micronized particles. To investigate the effect of precipitation pressure on the formation of phospholipid microparticles, several experiments were carried out. Only pressure was varied while other parameters were held constant. Experiments (1, 2, 3 and 4) were conducted at pressures of 9, 10, 11 and 13 MPa (308 K, solution flow rate 22.8 mL h^{-1} , solute concentration 15 wt.% and $\text{CO}_2/\text{solvent}$ molar ratio 60). Fig. 4 shows the SEM images of micronized phospholipid powders. Images exhibit spherical and aggregated particles with mean diameters varying from 5 to 50 μm . However, for lower precipitation pressure, particles seem to fuse together in large aggregates. This phenomenon was not observed for higher pressure experiments. In these cases, particles were less aggregated. All things considered, within the range of the study, microparticle size and morphology were not significantly influenced by pressure value. The same conclusions have already been highlighted by Magnan et al. [52] (308 K; and pressure range from 8 to 11 MPa), Badens et al. [53] (temperature range from 303 to 323 K; and pressure range from 8 to 12 MPa) and Kunastitchai et al. [65] (temperature range from 304 to 333 K; and pressure range from 8.5 to 10.5 MPa). On the contrary, Li et al. [51] (temperature range from 303 to 313 K; pressure range from 8 to 12 MPa) found that the higher the pressure, the larger the particle size of micronized

Table 1
Experimental conditions of the SAS process ($T = 308$ K and solution flow rate = 22.8 mL h^{-1}).

Ex. N°	P/MPa	$\text{CO}_2/\text{solvent}$ molar ratio	Solute concentration/wt.%	$\text{CO}_2/\text{lecithin}$ weight ratio
1	9	60	15	763
2	10	60	15	763
3	11	60	15	817
4	13	60	15	845
5	9	50	15	747
6	9	80	15	867
7	9	100	15	972
8	9	60	20	745
9	9	60	25	845

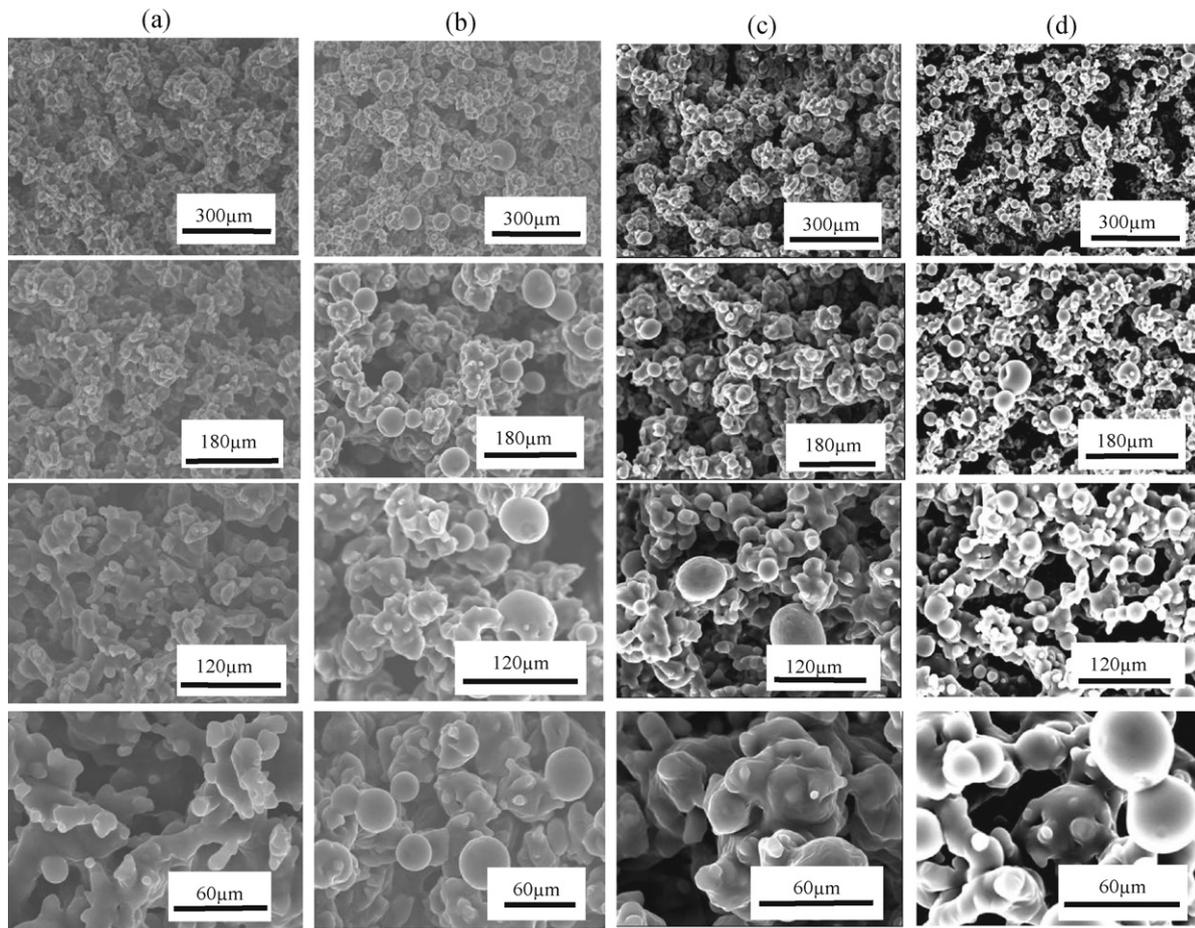


Fig. 4. SEM images of precipitated lecithin prepared respectively at different precipitation pressures: (a) 9 MPa. (b) 10 MPa. (c) 11 MPa. (d) 13 MPa (CO_2 /solvent molar ratio = 60 and solute concentration = 15 wt.%).

powder. According to Reverchon [66], pressure should not have any effect upon the particle size as long as its value corresponds to a monophasic domain of the solvent/anti-solvent mixture. The monophasic domain is reached for pressures larger than the asymptotic value of the liquid-phase volume expansion curve. Curves have been drawn by Badens et al. [53] for an ethyl alcohol/supercritical CO_2 mixture. It appears that, at 308 K, the asymptotic value of the pressure is estimated at 7.5 MPa. Thus, between 9 and 13 MPa, pressures are higher than the asymptotic value. Since no perceptible influence of pressure upon particle size has been observed here, our results are consistent with this tendency.

Influence of precipitation pressure on liposome size. The effect of the precipitation pressure of the SAS process on liposome produced by hydration of the micronized material has been studied. Size distribution curves are showed in Fig. 5. Table 2 provides details about size distribution curves. According to Fig. 5, size distribution curves are bimodal. There are two main populations of liposomes. The main one has a medium diameter range between 0.1 μm and 1 μm . The second one is more dispersed with a medium diameter range between 8 μm and 500 μm . According to Table 2, increasing pressure leads to decrease the proportion of small liposomes (diameter

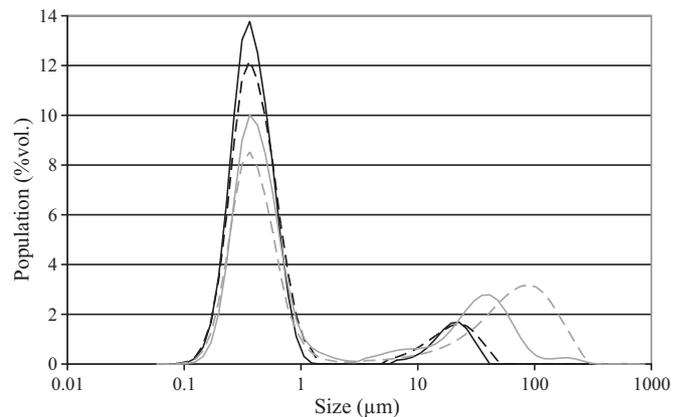


Fig. 5. Influence of pressure on particle size distribution curves of liposomes: — 9 MPa; --- 10 MPa; 11 MPa; - · - 13 MPa (CO_2 /solvent molar ratio = 60 and solute concentration = 15 wt.%).

Table 2

Cumulative volume percents of liposome populations presented in Fig. 5.

	$d (\Sigma\%) < 1.06 \mu\text{m}$	$1.06 \mu\text{m} < d (\Sigma\%) < 10.48 \mu\text{m}$	$10.48 \mu\text{m} < d (\Sigma\%)$
$P = 9 \text{ MPa}$	89.34	1.41	9.25
$P = 10 \text{ MPa}$	85.37	3.69	10.94
$P = 11 \text{ MPa}$	67.49	6.94	25.57
$P = 13 \text{ MPa}$	58.79	3.90	37.31

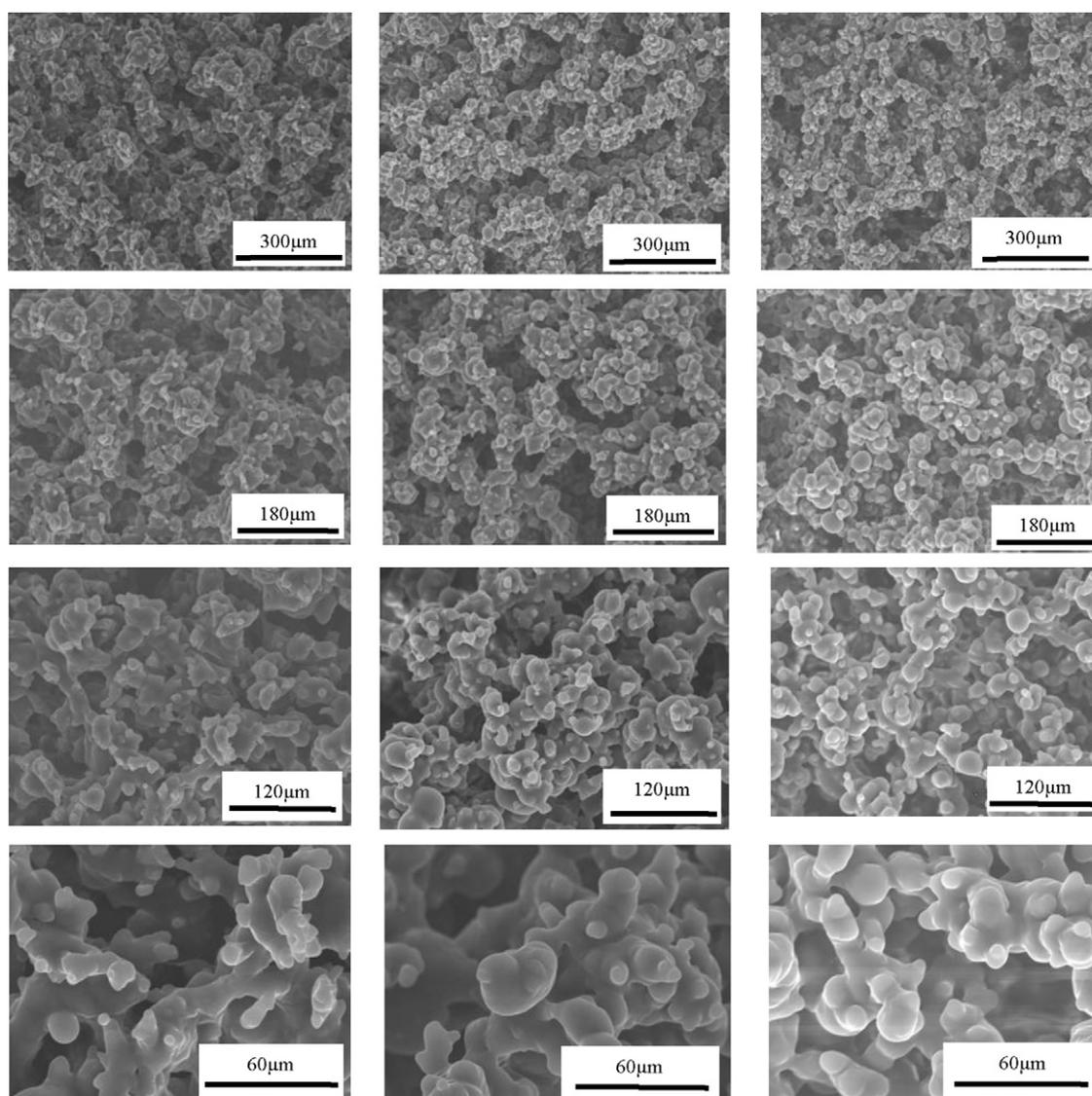


Fig. 6. SEM images of precipitated lecithin prepared respectively at different $\text{CO}_2/\text{solvent}$ molar ratios: (a) 60. (b) 80. (c) 100 ($P=9$ MPa and solute concentration = 15 wt.%).

below $1 \mu\text{m}$) and increase the proportion of big liposomes (above $10 \mu\text{m}$). Population of medium liposomes (diameter between 1 and $10 \mu\text{m}$) is not influenced by variations of pressure and remains at low proportions. To explain these results, one may consider that liposomes are formed at ambient pressure by hydration of the micronized phospholipid powder under stirring. Since hydration and stirring are performed in the same conditions for each experiment, one may assume that the properties of the phospholipids powder have significant influence on liposome formation. But, pressure was found to have only little effect on phospholipid micronization. Lastly, the use of a high stirring speed ($11\,000 \text{tr min}^{-1}$) may explain these results. For such high speeds, the stirring tends to form an homogeneous mixture of phospholipids in water whatever the characteristics of the phospholipid powder. However, the positive effect of the SAS process has to be taken into

account: lecithin material is finely divided which enhances liposome formation and significant difference are noted where the raw lecithin is processed with the Bangham method. For the following investigations, the lower operating conditions (9 MPa) are chosen from an energetic/effectiveness point of view.

3.1.2.2. Influence of $\text{CO}_2/\text{solvent}$ molar ratio. Influence of $\text{CO}_2/\text{solvent}$ molar ratio on micronized particles. Lecithin was precipitated at various $\text{CO}_2/\text{solvent}$ molar ratios from 50 to 100 (308 K, 9 MPa, solute concentration 15 wt.% and solution flow rate 22.8 mL h^{-1}), corresponding to the experiments 1, 5, 6 and 7. Either thermodynamic or kinetic influences on the precipitation are expected varying the $\text{CO}_2/\text{solvent}$ molar ratio. SEM images are shown in Fig. 6. It appears that for a $\text{CO}_2/\text{solvent}$ molar ratio of 50 (Fig. 7), processed lecithin is not well micronized, i.e. particles are not finely divided.

Table 3

Cumulative volume percents of liposome populations presented in Fig. 8.

	$d (\Sigma\%) < 1.06 \mu\text{m}$	$1.06 \mu\text{m} < d (\Sigma\%) < 10.48 \mu\text{m}$	$10.48 \mu\text{m} < d (\Sigma\%)$
$\text{CO}_2/\text{solvent}$ molar ratio = 60	89.34	1.41	9.25
$\text{CO}_2/\text{solvent}$ molar ratio = 80	81.03	3.40	15.57
$\text{CO}_2/\text{solvent}$ molar ratio = 100	62.38	3.65	33.97

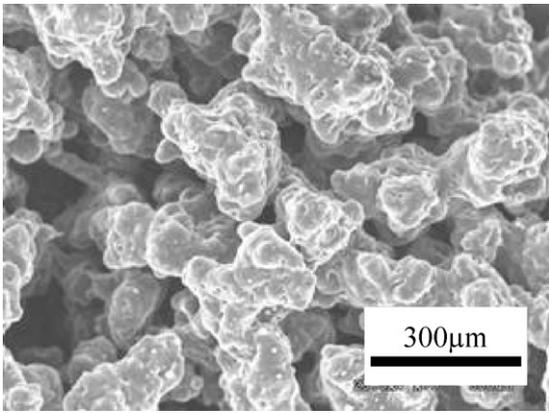


Fig. 7. SEM image of precipitated lecithin prepared with a CO_2 /solvent molar ratio of 50 ($P=9\text{ MPa}$, solute concentration = 15 wt.%).

Above 50, the value of the CO_2 /solvent molar ratio does not influence particle size and morphology any more; then, micronized material was composed of nearly spherical particles sizing about $20\ \mu\text{m}$ constituting aggregates. Generally, a low CO_2 /solvent molar

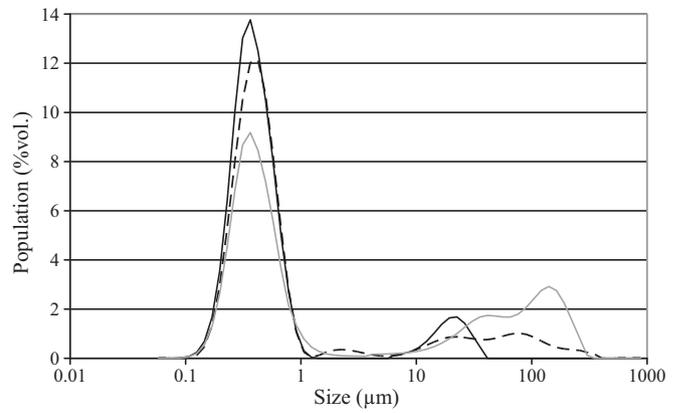


Fig. 8. Influence of CO_2 /solvent molar ratio on particle size distribution curves of liposomes: — 60; --- 80; ——— 100 ($P=9\text{ MPa}$ and solute concentration = 15 wt.%).

ratio would tend to slow down mass-transfer kinetics between the dispersed liquid phase and the continuous supercritical phase; and consequently, micronized particles are expected to be bigger [53]; but, this tendency was not observed in this study. A fixed

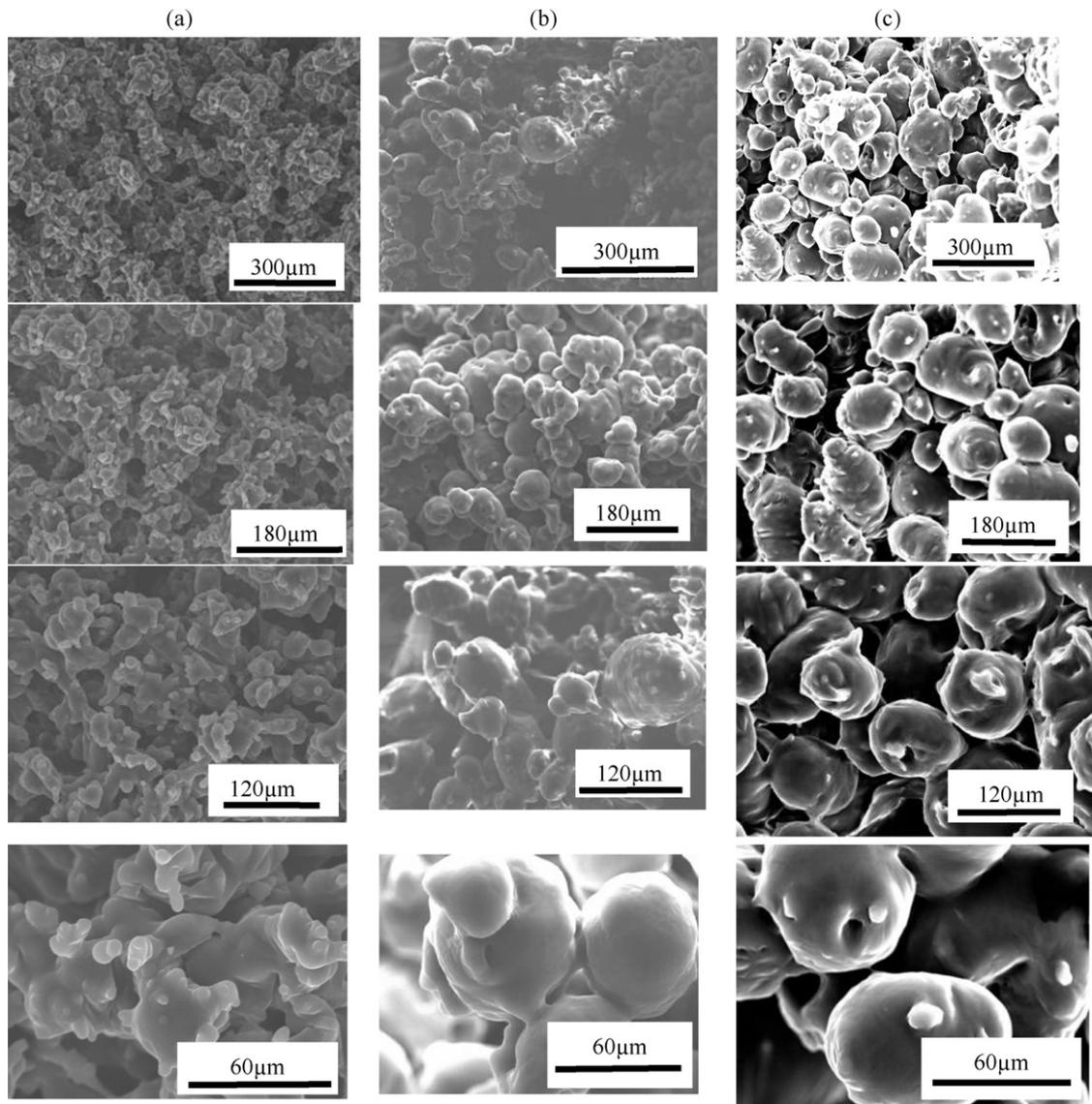


Fig. 9. SEM images of precipitated lecithin prepared respectively at different lecithin concentrations: (a) 15 wt.%. (b) 20 wt.%. (c) 25 wt.% ($P=9\text{ MPa}$ and CO_2 /solvent molar ratio = 60).

CO₂/solvent molar ratio of 60 was taken as setting in the following experiments.

Influence of CO₂/solvent molar ratio on liposome size. The influence of the CO₂/solvent molar ratio of the SAS process on size distribution curves of liposomes is shown in Fig. 8. Table 3 provides details about size distribution curves. According to Fig. 8, size distribution curves are bimodal. The main population of liposomes is between 0.1 and 1 μm. The other population presents liposome sizes above 10 μm. According to Table 3, increasing the CO₂/solvent molar ratio brings about an increase of the proportion of bigger liposomes (diameter above 10 μm). Once again, it is difficult to draw some conclusions because micronization results were not significant. One may assume that high stirring speed tends to form an homogeneous mixture of phospholipids in water whatever the characteristics of phospholipid powder. Thus, there is no significant difference between liposome sizes but results are quite different when the raw lecithin is processed with the Bangham method. One may conclude that the effect of the SAS process is beneficial to the formation of liposomes.

3.1.2.3. Influence of solute concentration. Influence of solute concentration on micronized particles. Lecithin was also precipitated from organic solution at various lecithin concentration (experiments 1, 8 and 9) in the range from 15 to 25 wt.% (308 K, 9 MPa, solution flow rate 22.8 mL h⁻¹ and CO₂/solvent molar ratio 60) to investigate the characteristics of produced phospholipid microparticles. A significant influence of lecithin concentration on the particle size and morphology was observed on SEM images (Fig. 9). The mean particle size was increased from 20 μm to 60 μm with increasing lecithin concentration from 15 to 25 wt.%. Moreover, it appears that higher concentrated solution leads to more distinct spherical objects. Li et al. [51] observed that different solute concentrations lead to significant distinct morphology of particles (agglomerated large blocks, flake structures besides particles, connected particles and massive blocks besides irregular microparticles). Kunastitchai et al. [65] noted that raising concentration of the spraying solution up to 16.7 wt.% initiated crystallization (cubic- and rod-like shape). In our case, only size of microparticles is affected by solute concentration. In crystallization, influence of supersaturation on crystal growth is well-known. On a plot of crystal size as a function of supersaturation, there are two domains: the first one for low supersaturation where nucleation is the prevailing mechanism and the second one, for high supersaturation, where Ostwald ripening becomes the major mechanism of crystal growth. Thus, high levels of supersaturation may entail the formation of bigger crystals. Reverchon et al. [67] propose another explanation to this phenomenon. When diluted solutions are injected, saturation and precipitation of solute is reached very late during the droplet expansion process; therefore, nucleation is the prevailing mechanism and thus smaller particles are formed. When concentrated solutions are used, the precipitation of the solute is obtained early during the expansion process and the growth process is the prevailing mechanism thus producing larger particles.

Influence of solute concentration on liposome size. Fig. 10 reveals size distribution curves of liposome as a function of the lecithin concentration used in the SAS process. Table 4 provides details about size distribution curves. According to Fig. 10, size distri-

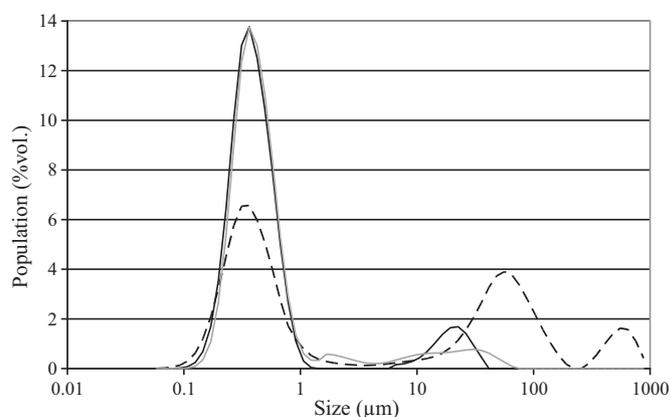


Fig. 10. Influence of solute concentration on particle size distribution curves of liposomes: — 15 wt.%; --- 20 wt.%; ——— 25 wt.% ($P=9$ MPa and CO₂/solvent molar ratio = 60).

bution curves are bimodal (solute concentrations of 15 wt.% and 25 wt.%) or trimodal (solute concentration of 20 wt.%). For solute concentration of 15 and 25 wt.%, size distribution of liposomes are quite equal (Table 4). As concerns the solute concentration of 20 wt.%, size distribution curve shows more dispersed liposomes than for the other concentrations. Thus, influence of solute concentration on liposome size is not meaningful. One may assume that high stirring speeds tend to form an homogeneous mixture of phospholipids in water masking the characteristics of phospholipid powders. Thus, there is no significant difference between liposome sizes but results are quite different when the raw lecithin is processed with the Bangham method. One may conclude that the effect of the SAS process has to be taken into account.

3.1.2.4. Conclusion. In all cases, it appears that the SAS process enables the formation of finely divided microparticles of lecithin. Variations of pressure and CO₂/solvent molar ratio do not influence the micronization process. Conversely, increasing solute concentration leads to the formation of bigger phospholipid microparticles. Lastly, size distribution of liposomes are rather similar. One may assume that the use of high stirring

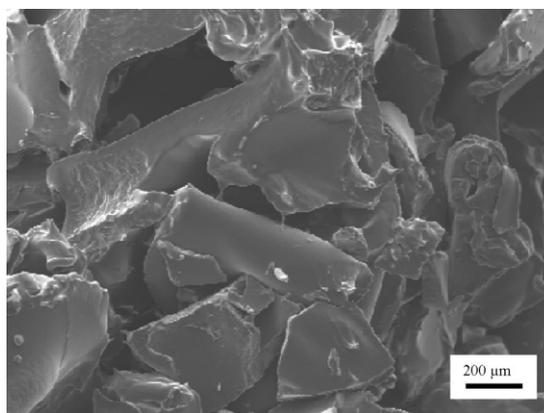


Fig. 11. SEM image of the raw lecithin.

Table 4

Cumulative volume percents of liposome populations presented in Fig. 10.

	$d(\Sigma\%) < 1.06 \mu\text{m}$	$1.06 \mu\text{m} < d(\Sigma\%) < 10.48 \mu\text{m}$	$10.48 \mu\text{m} < d(\Sigma\%)$
Solute concentration = 15 wt.%	89.34	1.41	9.25
Solute concentration = 20 wt.%	51.71	4.46	43.82
Solute concentration = 25 wt.%	86.79	6.31	6.90

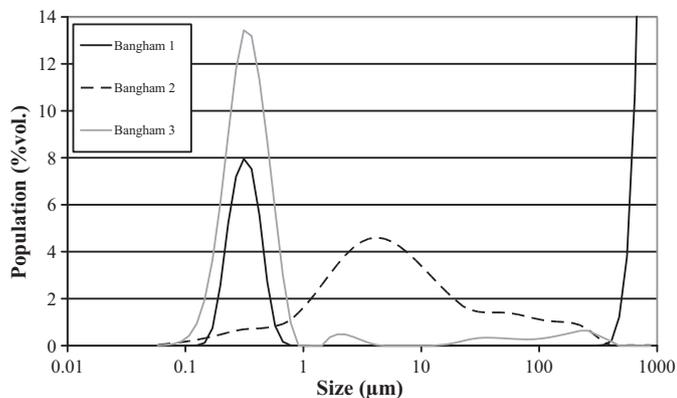


Fig. 12. Particle size distribution curves of liposomes prepared from lecithin processed with the Bangham method.

speed yields to the formation of quite similar liposomes. But, the effect of the SAS process is not cancelled with the stirring because results are quite different when the raw lecithin is processed with the Bangham method. Formed liposomes are the result of both, the lecithin processing with the SAS process and the hydration/stirring step. Consequently, experimental conditions can be chosen according to the point of view of fluid consumption and processing time: $P=9$ MPa; CO_2 /solvent molar ratio = 60 and solute concentration = 15 wt.% ($T=308$ K and solution flow rate = 22.8 mL·h⁻¹).

3.2. Bangham process

Liposomes have been produced from lecithin processed with the Bangham method. Fig. 11 shows a SEM image of the raw material. Three experiments have been performed in the same conditions (respectively noted Bangham 1, Bangham 2 and Bangham 3). Size distribution curves are presented in Fig. 12. Table 5 provides details about size distribution curves. Fig. 12 clearly shows that the Bangham method is not reproducible for the given experimental conditions. Reproducibility of the SAS process has been tested and is validated as shown in Fig. 13.

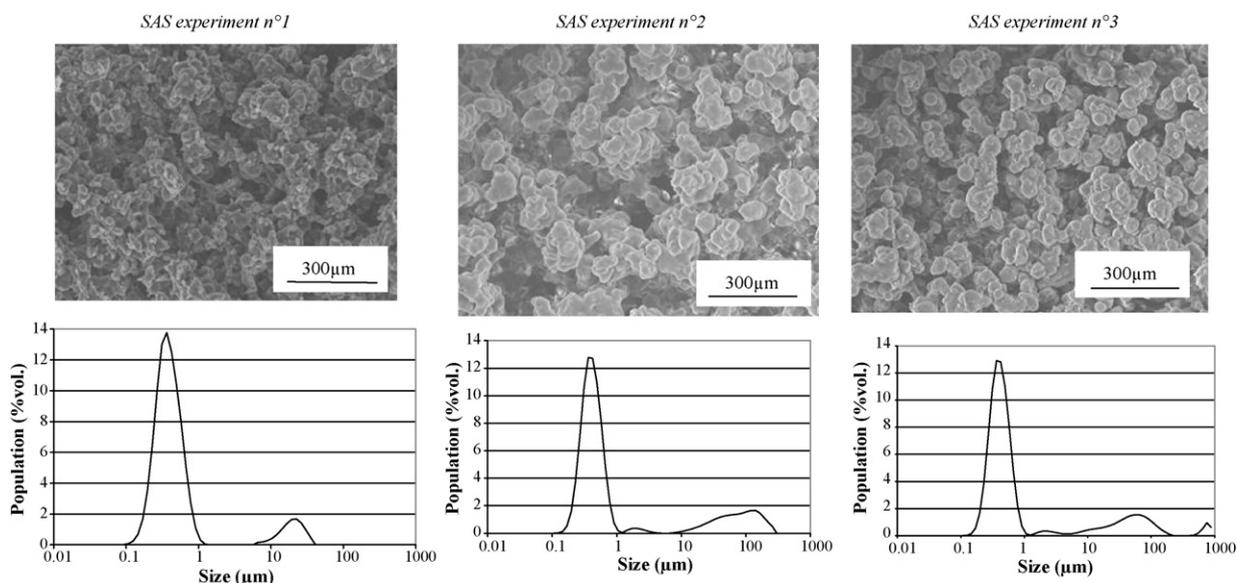


Fig. 13. Reproducibility trials for the SAS process ($P=9$ MPa, CO_2 /solvent molar ratio = 60, solute concentration = 15 wt.%): SEM images of processed phospholipids and size distribution curves of liposomes.

Table 5

Cumulative volume percents of liposome populations presented in Fig. 12 (Bangham method).

	d ($\Sigma\%$) < $1.06 \mu\text{m}$	$1.06 \mu\text{m} < d$ ($\Sigma\%$) < $10.48 \mu\text{m}$	$10.48 \mu\text{m} < d$ ($\Sigma\%$)
Bangham 1	40.62	0.00	59.38
Bangham 2	10.30	58.66	31.03
Bangham 3	90.47	2.09	7.44

It has to be noted that the Bangham method may yield better results concerning liposome size distribution (unimodal size distribution) when employed with another solvent such as chloroform. However, in this study, the Bangham method has been implemented with absolute ethyl alcohol in order to be compared with the SAS method.

3.3. Microscopic observations of liposomes: SAS process/Bangham process

Liposome images are presented in Fig. 14. It appears that the SAS process enables the formation of spherical and micrometric liposomes (Fig. 14a). Conversely, Bangham liposomes are ellipsoidal liposomes and are taller than the ones formed with the SAS process. Thus, liposomes formed with the SAS process are easier to characterize. The SAS process provides finely divided phospholipid microparticles from raw lecithin which is determining for liposome formation.

3.4. Control of residual solvent – SAS process/Bangham process

Presence of organic solvent in the processed material was assessed thanks to an InfraRed method. For each samples, the residual solvent level is estimated comparing the intensity of the alcohol peak with the intensity of the carboxyl group (lecithin) peak, respectively at 1058 cm⁻¹ and 1736 cm⁻¹. Table 6 presents the values of $h_{1058 \text{ cm}^{-1}}/h_{1735 \text{ cm}^{-1}}$ for samples with known amount of absolute ethyl alcohol (respectively 1, 5.5 and 10 wt.%). For processed material with the SAS process, values of $h_{1058 \text{ cm}^{-1}}/h_{1735 \text{ cm}^{-1}}$ are comprised between 0.76 and 1.1; and for the conventional method, the value is about 1.2. According to Table 6, the content of residual solvent for samples processed with the SAS method is about 10 wt.%; and, for the conventional technique, the remaining

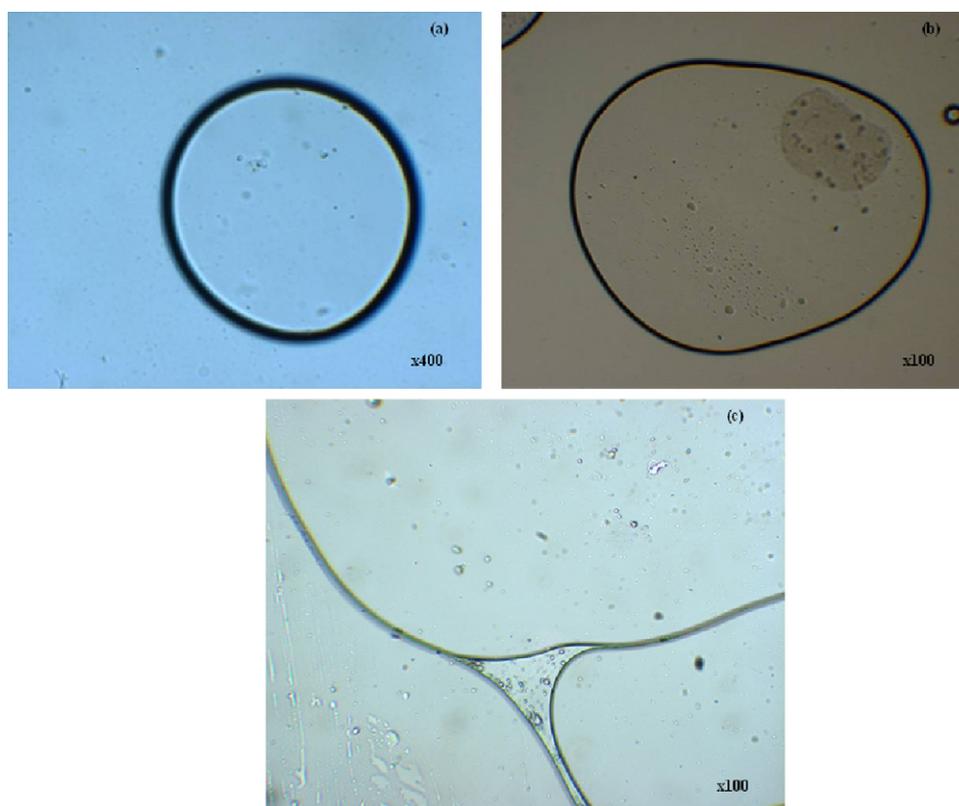


Fig. 14. Phase contrast optical microscopic images of liposomes produced with (a) the SAS method ($P=9$ MPa, $T=308$ K, CO_2 /solvent molar ratio = 60, solute concentration = 15 wt.%, solution flow rate = 22.8 mL h^{-1}) and (b, c) the Bangham method.

Table 6

Height ratio of the carboxyl group (1735 cm^{-1}) and the alcohol group (1058 cm^{-1}) for three samples loaded with known amounts of ethyl alcohol.

Sample	$h_{1058\text{ cm}^{-1}}/h_{1735\text{ cm}^{-1}}$
Unprocessed lecithin S75	0.966
Lecithin loaded with 1 wt.% of absolute ethanol	1.004
Lecithin loaded with 5.5 wt.% of absolute ethanol	1.039
Lecithin loaded with 10 wt.% of absolute ethanol	1.095

quantity of solvent in the final product is higher than 10 wt.%. For the SAS process, better results may be expected considering data reported in the literature: the residual solvent level in materials processed with the SAS process is less than 1 wt.% [68]. However, it may be worth noting that the use of lecithin and ethyl alcohol raises some problems. First of all, because ethyl alcohol has a high boiling point; and secondly, because of the affinity of lecithin for ethyl alcohol. These two points may explain the level of residual solvent in the processed lecithin.

3.5. Liposome encapsulation efficiency – SAS process/Bangham process

Encapsulation efficiency of liposomes produced with the SAS process was investigated for given experimental conditions: solute concentration = 15 wt.%; $P=9$ MPa; CO_2 /solvent molar ratio = 60; $T=308$ K and solution flow rate = 22.8 mL h^{-1} . As concerns the SAS process, encapsulation efficiency values were comprised between 10 and 20%. This difference is due to measurement uncertainties. For the Bangham method, results were more stable: an encapsulation efficiency of 20% was measured. From these results, an assessment of the processes is not possible.

3.6. Liposome stability – SAS process/Bangham process

3.6.1. SAS process

Size measurements of liposomes have been carried out after storage. Liposome size distribution curves at $t=0$ and $t=1$ month are presented in Fig. 15 ($t=0$ stands for the day of liposome production). It appears that liposomes are not stable after one month of storage.

3.6.2. Bangham process

The same results have been observed with liposomes produced from lecithin processed with the Bangham method. Liposomes are not stable in suspension form.

3.6.3. Discussion

SAS and Bangham methods are similar concerning liposome stability. However, liposome stability is a complex issue. In a dispersed aqueous system, liposomes are damaged by hydrolysis or oxidation; and/or sedimentation, aggregation, or fusion of liposomes during storage [69]. Hydrolysis detaches the hydrophobic chains of ester bonds. Oxidation is more likely due to the presence of unsaturated chains. Gravity is the driving force of sedimentation. Aggregation is the result of attractive forces between phospholipid vesicles themselves and between the vesicles and the substrate. Fusion entails a structural rearrangement of two phospholipid bilayers to form a single one. Oxidation and hydrolysis of phospholipids dispersed in aqueous solutions depend on pH, ionic strength, temperature, composition of phospholipids, i.e. chemical parameters. Sedimentation, aggregation or fusion are also linked with liposome concentration, the structure of the dispersion (micelles, monolayer or multilayer liposomes) and biological parameters such as the nature of the buffer [70]. Therefore, cholesterol [71,72], charged lipids [72], polymers [73] or others are usually added to

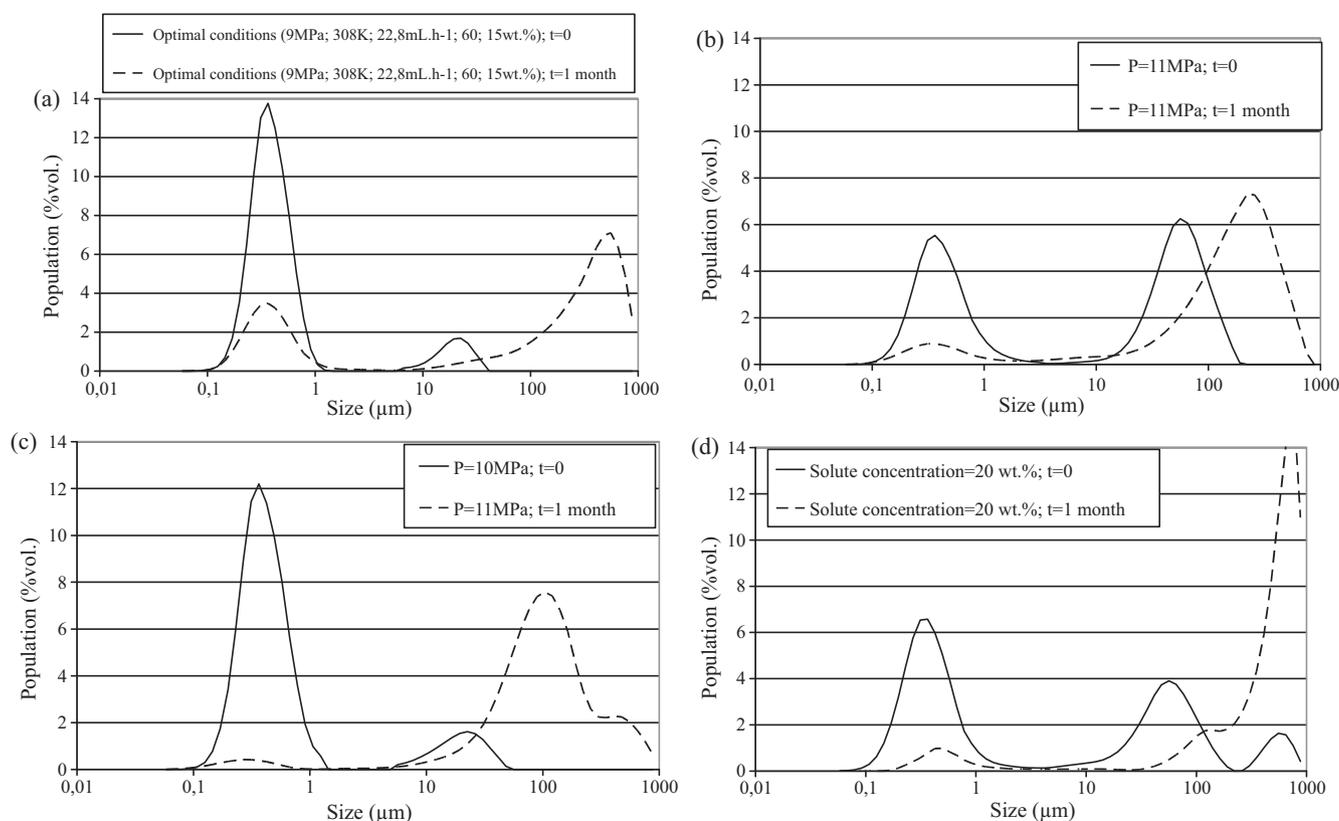


Fig. 15. Stability study on particle size distribution curves of liposomes produced from lecithin processed with the SAS process (experimental conditions are summed up in the caption).

phospholipids in order to increase liposome stability. Cholesterol is known to have three effects: (i) increasing the fluidity or microviscosity of the bilayer; (ii) reducing the permeability of the membrane to water soluble molecules; and (iii) stabilizing the membrane in the presence of biological fluids [74]. Moreover, the presence of charged lipids reduces the likelihood of aggregation after preparation of the liposomes [72]. Another possibility is to transform liposomes into a stable product thank to freeze drying by lyophilizer [75,76]. Today, freeze drying is the most frequently used method as a possible tool in scaling-up for liposome production and conservation.

4. Conclusion

Finally, two processes have been compared to produce liposomes: supercritical anti-solvent (SAS) process and Bangham process known as a conventional method. According to the results of this study, the use of the SAS process appears to be an efficient and environmentally-friendly process to produce liposomes. Efficient because liposome size distribution is included in the range of 0.1–100 μm (>80 cumulative volume percent) and encapsulation efficiency is about 20%, which corresponds to commonly expected values (to be used in drug carrier, a liposome size of 2–5 μm yields optimal benefit [77]); and environmentally-friendly because this technique enables to use “soft” organic solvents such as ethyl alcohol compared with the organic solvents used with conventional methods (isopropyl ether, diethyl ether, chloroform and methyl alcohol). Moreover, the SAS process is carried out under mild temperature conditions unlike the Bangham method (308 K for the SAS process and 323 K for the Bangham process) which constitutes an asset when liposomes are employed as DDS. In this study, investigations about influence of some experimental parameters of the SAS process (pressure, CO_2 /solvent molar ratio and solute concentra-

tion) have been carried out. If pressure and CO_2 solvent molar ratio have no influenced on micronized particles, variations of solute concentration significantly affect micronized material. Increasing solute concentration leads to the formation of bigger particles. Lastly, whatever the parameter studied, there is no significant difference between liposome size. An explanation may be that the use of high stirring speeds tends to form an homogeneous mixture of phospholipids in water masking the characteristics of phospholipid powders. However, the effect of the SAS process is not cancelled because results are quite different when the raw lecithin is processed with the Bangham process. To conclude, the effect of the SAS process is beneficial to liposome formation. Concerning the assessment of the two processes, encapsulation efficiency values are quite similar but microscopic images of liposomes are meaningful. Liposomes formed with the SAS process are spherical unlike Bangham liposomes which are ellipsoidal. Then, SAS liposomes are easier to characterize. Moreover, one may assume SAS liposomes are more stable than Bangham liposomes. Lastly, there are more scale-up issues with the Bangham method. Finally, the scope of this study has been exceeded by another issue: liposome stability in suspension form. Whatever the process employed, liposome stability remains a challenging task; all the more because liposome stability is linked with leakage of the encapsulated material. Recent advances in drug formulation [77] have shown that the use of phospholipid microparticle dry powder (called dry liposomes) mixed with drug enables to overcome the problem of liposome stability and drug encapsulation. Dry liposomes can be hydrated immediately before use or directly administrated (liposomes would form in the biological fluids of the body). Thus, the SAS process would meet the need of enhanced powder performance. However, in the case of the SAS process, lecithin micronized particles collected are not stable (spontaneous coalescence of the processed phospholipids upon contact with air) [33]. In the future, several key issues concerning

stability need to be addressed in order to find a route from theory to clinical reality.

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