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# Elaboration of Lutein-Loaded Nanoliposomes Using Supercritical CO<sub>2</sub>

Mathieu Martino,\* Adil Mouahid, Paolo Trucillo, and Elisabeth Badens

A batch process for producing lutein-loaded liposomes using supercritical CO<sub>2</sub> is studied. The effects of the variation of pressure (10 and 15 MPa), temperature (308, 313, and 318 K), and lutein to lipid ratio (0.5 and 1 wt%) on the liposome average size and size distribution are investigated, as well as on the encapsulation efficiency (EE) of lutein. This process is worked in a repeatable manner and is allowed the production of nanoliposomes with mean diameters (MDs) ranging from 65 ± 33 to 77 ± 40 nm, obtaining lutein EEs ranging from 82.1 ± 3.7% to 91.9 ± 2.9%. Temperature, pressure, and lutein to lipid ratio seem to have no impact on size, size distribution, and EE on formed liposomes. The use of low temperatures and low pressures allows the obtainment of liposomes with diameters less than 100 nm and limits the process energy cost. Moreover, the supercritical CO<sub>2</sub>-assisted batch process effectively encapsulates lutein into liposome, an antioxidant molecule used for the prevention of retinal damage. Liposomes formed by this supercritical process have the desired characteristics for human target delivery.

**Practical applications:** This work on the optimization of a process for developing liposomes in a supercritical environment has applications in medicine. Indeed, the liposomes formed with this process are nanoliposomes with a size of less than 80 nm. In addition, excellent lutein EEs (hydrophobic molecules) show that the liposomes formed constitute excellent coating matrices for the protection of active ingredients. These reasons make these liposome matrices applicable in nanomedicine (injection of sensitive drugs requiring protection before injection). The elaboration process also makes it possible to form liposomes with desired properties by changing pressure, temperature, or lecithin concentration. Therefore, this work focuses on the properties of liposomes as a function of the operating conditions.

## 1. Introduction

One of the major challenges in therapy is to find a drug carrier that better protects therapeutic molecules for targeted drug delivery.<sup>[1,2]</sup> In addition to this challenge, environmental and health issues are now also being taken into consideration.<sup>[3]</sup> These issues led to the emergence of new techniques for the development of Drug Delivery Systems (DDS), limiting the use of organic solvents.<sup>[4,5]</sup> For these reasons, several processes using supercritical fluids have been developed.<sup>[6–10]</sup> Drug formulation by using supercritical fluid technology, especially supercritical carbon dioxide (SC-CO<sub>2</sub>), has several advantages, such as the reduction of the quantity of organic solvent required. Another advantage is that CO<sub>2</sub>-based processes are more compact since carbon dioxide is gaseous at ambient conditions; therefore, the final product is spontaneously separated from CO<sub>2</sub> by simple depressurization.

In parallel, numerous studies have been performed on the use of liposomes as encapsulation vectors.<sup>[11]</sup> Liposomes are biodegradable vesicles composed of phospholipids, thus they mimic cell membranes with which they can fuse to deliver the encapsulated drug.<sup>[12]</sup> This liposome/cell fusion allows both hydrophilic and hydrophobic drug delivery.<sup>[13]</sup> Therefore, liposomes behave as protective agents for

the encapsulated pharmaceutical active substances once administered. This protection prevents enzymatic degradation and drug elimination by the immune system.<sup>[14]</sup> Liposomes are thus promising vector agents for several medical therapies. Nevertheless, a limitation for their use as drug carriers for gene or cancer therapy is their size. Indeed, particle size is a key characteristic for a vesicle/particle cellular internalization. A particle size of up to 5 microns can undergo cell internalization, but the process is more rapid for particles with a size smaller than 100 nm. It is commonly stated that the recommended diameter of particles for treating cancer is in the range 10–100 nm, and nanoparticles in the low tens of nanometer range enter more effectively using pathways of cell internalization, limiting degradation.<sup>[15]</sup> There is then a real interest for forming submicron-sized liposomes or nanoliposomes.

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In this context several studies have been conducted during these past decades on the encapsulation of drugs into liposomes.<sup>[16–19]</sup> More recently, particular interest was focused on the use of supercritical-assisted processes.<sup>[20–30]</sup> Therefore, the combined advantages of liposome encapsulation and supercritical fluid processes allowed the development of biomimetic and biodegradable small vesicles for drug encapsulation using a green process, and allowed a good control of their characteristics.<sup>[31]</sup>

The aim of this study is to investigate the temperature, the pressure, and the lutein to lipid ratio influences on liposome properties using a supercritical CO<sub>2</sub>-assisted process operated in a batch mode. In order to be able to compare the liposomes formed in terms of encapsulation efficiency (EE), a molecule of therapeutic interest for ocular delivery—as well as of nutritional interest—was encapsulated: Lutein.<sup>[32]</sup> This molecule has antioxidant properties, used in particular for the prevention of retinal damage<sup>[33]</sup> and cataracts.<sup>[34]</sup> Lutein also helps to reduce the risk of cardiovascular diseases.<sup>[35]</sup> The encapsulation of lutein into liposomes using supercritical carbon dioxide has already been investigated in several studies,<sup>[36–38]</sup> where high EEs were reported (over 90% and up to 97% for these studies). The batch process performance was evaluated in terms of mean size of the liposomes formed, particle size distribution and lutein EE. The effects of pressure (10–15 MPa), temperature (308–318 K), and drug to lipid ratio (0.5–1 wt%) were studied. The impact of these factors on the diameter of the liposomes formed, size distribution, EE, and process repeatability is discussed.

## 2. Experimental Section

### 2.1. Materials

L- $\alpha$ -phosphatidylcholine from egg yolk (PC,  $\approx$ 60% purity) was purchased from Sigma-Aldrich, France, and used as a source of phospholipids. Absolute ethanol (purity 99.8%) was provided by VWR, France. Carbon dioxide (>99.7% purity) was purchased from Air Liquide, Vitrolles, France. Distilled water was directly produced in our laboratories. Lutein was provided by Shaanxi Superior Bio Technology Co., a Natural Herbal Extract Manufacturer in China, and was obtained from the extraction of marigold flowers.

### 2.2. Feed Solution Preparation

The phospholipid feed solution was prepared in a water/ethanol solution (79/21% w/w). Egg lecithin (0.4 g) and lutein (0.002–0.004 g depending on the desired lutein/lecithin ratio) were dissolved in ethanol (61.5 g). The solution was stirred with a magnetic stirrer until lutein and lecithin were completely dissolved. Distilled water (228.5 g) was then added to the solution (ethanol with lecithin and lutein) and mixed again. A lecithin mass concentration of 0.14% lecithin and lutein/lecithin mass ratios ranging from 0.5% to 1% by mass were obtained in the feed solution (307.5 mL).

### 2.3. Experimental Set-up

The laboratory scale set-up is shown in **Figure 1**. The stainless steel high pressure autoclave (A) was provided by Top Industrie, France. The autoclave had a volume of 0.48 L for an internal diameter of 0.06 m and a length/diameter ratio of 2.83. It was equipped with a water jacket to allow for constant temperature experiments when connected to a heating bath circulator (E2). The maximal operating temperature and pressure were 423 K and 36 MPa. Inside the autoclave, the stirring was performed thanks to a magnetic stirrer (M). Internal temperature was measured with a type K thermocouple inserted in a thermowell.

The CO<sub>2</sub> was cooled to 273.15 K by a cold bath circulator (E1) to ensure the CO<sub>2</sub> liquid state, it was then pumped by a liquid high-pressure pump (HPP6/LGP50, Separex, France) to reach a pressure of 7.8 MPa in the autoclave (A). Once the CO<sub>2</sub> had been introduced and stabilized at the desired temperature (308, 313, 318 K), the feeding solution was then injected at a flow rate of 10.8 mL min<sup>-1</sup> by a high-pressure liquid pump P2 (Gilson 305, France) into the autoclave under pressure and constant agitation (800 rpm). The feed solution passed through a stainless steel frit (3  $\mu$ m porosity) placed on the top of the autoclave. CO<sub>2</sub> and feeding solution lines were preheated by a hot circulator bath (E2) before their introduction in the autoclave. Once the amount of prepared feeding solution was totally introduced in (A), CO<sub>2</sub> was again injected in (A) in order to reach the desired working pressure (10 and 15 MPa). The mixture in the autoclave (water, CO<sub>2</sub>, ethanol, phospholipids, and lutein) was then left to stir for 30 min.

At the end of this mixing step, the stirring was stopped and the micrometric valve MV1, located at the bottom (exit) of the autoclave, was opened in order to recover the liposomal suspension. The outlet of the micrometric valve was connected to a 1/16" high-pressure stainless steel tube (internal diameter of 0.5 mm) for discharging into a cold solvent trap at 273 K. The solvent trap was composed of a hermetically closed container permanently cooled by a 273 K ice bath. The liposomal suspension was formed at the exit of the tube during the depressurization. In the solvent trap, CO<sub>2</sub> became gaseous and spontaneously separated from the liposomal suspension. The CO<sub>2</sub> flow rate was measured at the outlet of the trap by a flowmeter before venting. During the solution racking time, the pressure in the autoclave was kept at constant pressure (10–15 MPa) thanks to P1 automatic pressure regulation. When only CO<sub>2</sub> flowed out of the MV1, P1 was stopped and the depressurization of the autoclave was carried out. The liposomal suspension formed was recovered and stored at 4 °C in a dark place to avoid lutein degradation. The resulting liposomal suspension also contained ethanol. The ethanol was removed by evaporation with a rotary evaporator (Laborota 4000, Heidolph, Germany) without degradation of the formed liposomes.

A simplified representation of the formation of liposomes is given in **Figure 2**. The injection of the aqueous-organic solution into the autoclave loaded with supercritical CO<sub>2</sub> was followed by the formation of a CO<sub>2</sub>/water or water/CO<sub>2</sub> emulsion, depending on the operating conditions and on the global composition.<sup>[39]</sup> During depressurization, due to CO<sub>2</sub> release, a water-in-water emulsion was then formed. This corresponds to the organization of the phospholipids in the form of liposomes allowing the encapsulation of the therapeutic molecule.<sup>[39]</sup>

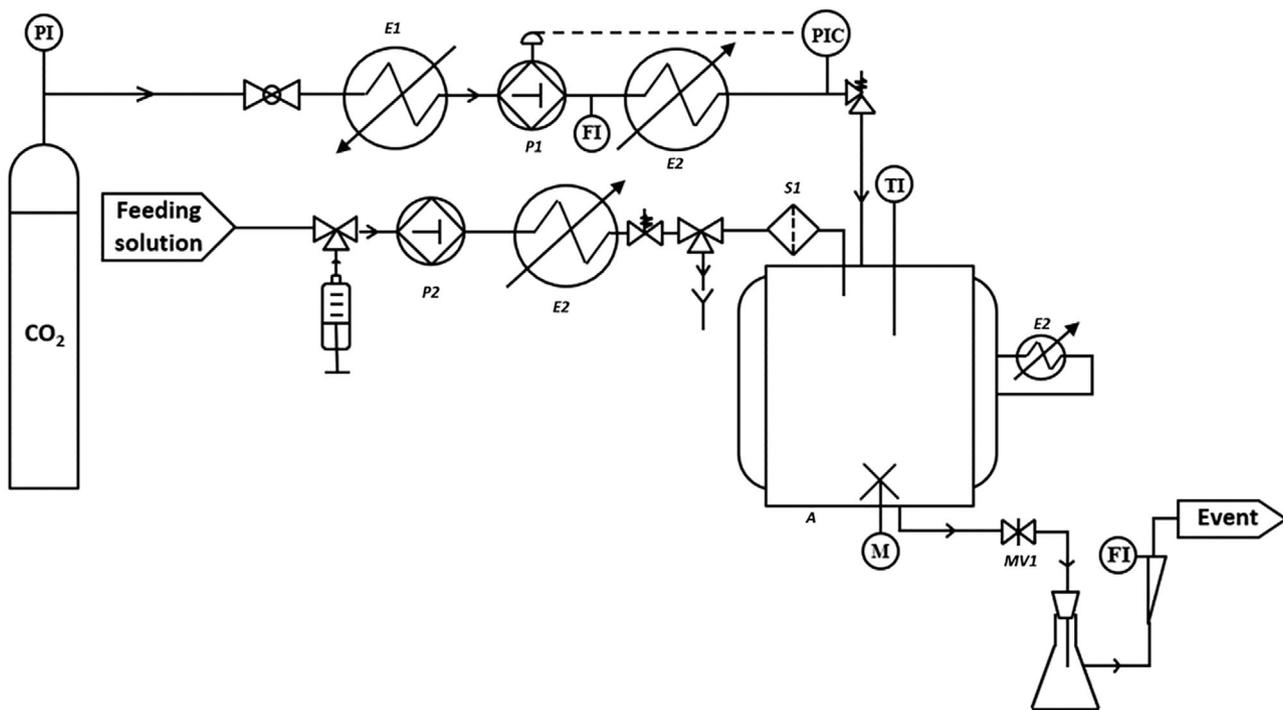


Figure 1. Experimental set-up of liposome batch formation process.

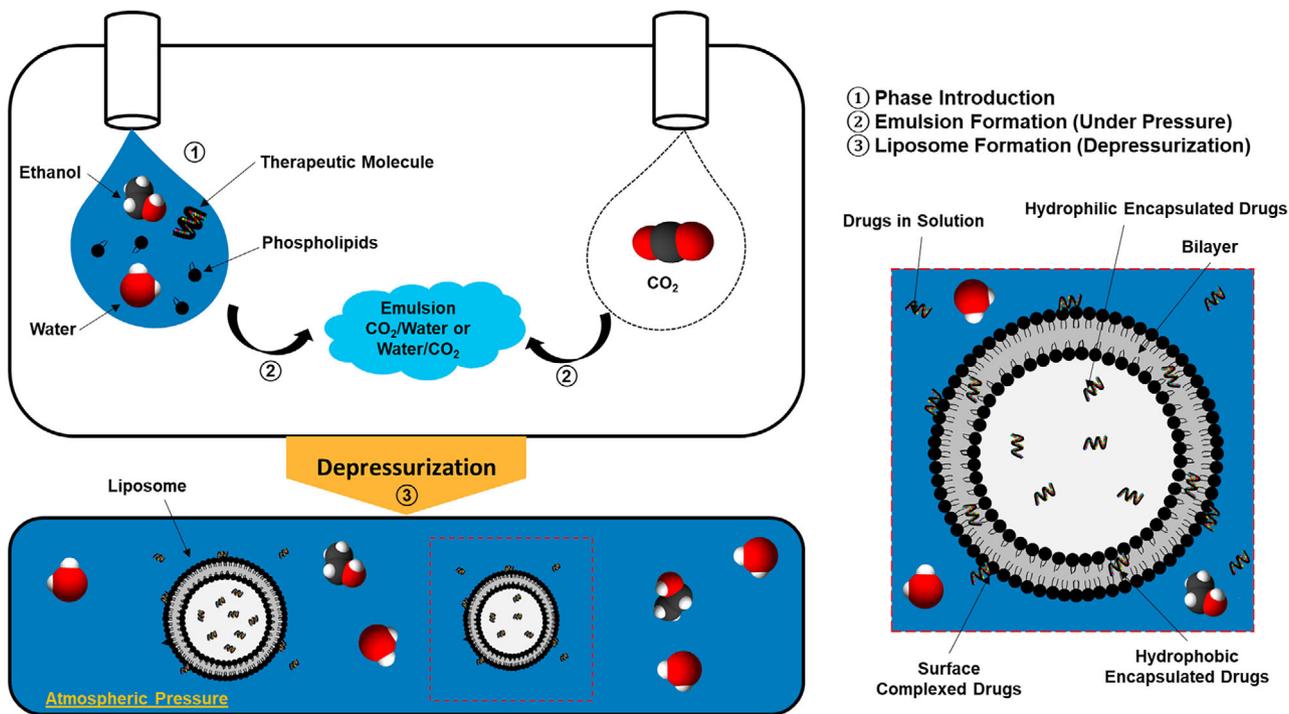


Figure 2. Schematic representation of batch process for forming liposome in supercritical.

**Table 1.** Experimental conditions (pressure, temperature, drug to lipid ratio) for the study of operating conditions influence.

Test name	Pressure[MPa]	Temperature[K]	Drug to lipid ratio [% w/w]
Lut1	10	313	1
Lut1'	10	313	1
Lut1''	10	313	1
Lut2	10	308	1
Lut3	10	318	1
Lut4	15	308	1
Lut5	15	318	1
Lut6	10	308	0.5
Lut7	10	318	0.5
Lut8	15	308	0.5
Lut9	15	318	0.5

## 2.4. Operating Conditions

In order to investigate the influence of the operating conditions on the properties of the liposomes formed, 11 experiments were conducted. These experiments were performed with varying operating pressure, temperature, and drug to lipid ratios. The repeatability has also been investigated. The operating conditions of the experimental campaign are summarized in **Table 1**.

The liposomal suspensions obtained at these conditions were analyzed (liposome mean particle size, particle size distribution, and EE) in order to determine the optimal operating conditions.

## 2.5. Characterization

### 2.5.1. Liposome Size

Liposomal suspensions were characterized by a Dynamic Light Scattering (DLS) instrument (Zetasizer nano S, UK) with a measuring range of 0.3–10,000 nm. These analyses allow the determination of particle mean size and particle size distribution of liposomes in aqueous suspensions. Liposome size was characterized by their Mean Diameter (MD) with associated Standard Deviation (SD). The particle size distribution was also characterized by the PolyDispersity Index (PDI) with its associated SD. The light source of the Zetasizer instrument is a He-Ne laser (4 mW, 633 nm). In this work, for each sample, five measurements were performed at 298 K in a 10 mm quartz glass cell (Hellma, Germany). An average was then taken for all parameters.

The results obtained were compared in terms of size and size distribution for the liposomes formed under different operating conditions. The error bars were determined by averaging the five measurements and calculating the SD of the measurements.

### 2.5.2. Encapsulation Efficiency

EE was determined by UV–visible spectrometry. First, the liposomal solution was analyzed in UV–visible spectrometry to determine the total amount of lutein (encapsulated and free). Then a fraction of the liposomal suspension was filtered through a

**Table 2.** Encapsulation efficiency (EE) value obtained during reproducibility tests.

Test	Mean diameter (MD) ± SD [nm]	EE ± SD [%]
Lut 01	67 ± 36	83.8 ± 1.1
Lut 01bis	69 ± 36	85.6 ± 1.4
Lut01 ter	71 ± 37	85.8 ± 1.0

regenerated cellulose ultrafiltration membrane with a nominal molecular weight limit (NMWL) of 10,000 Da (Millipore, USA). This filtration was carried out under a compressed air flow in order to maintain a constant filtration pressure of 0.2 MPa. Once filtered, the supernatant was passed through UV–visible spectrometry to determine the concentration of unencapsulated lutein. All spectrometric analyses were performed in 10 mm quartz glass cells (Hellma, Germany) at a wavelength of 380 nm. The maximum wavelength of absorption was determined by scanning in the wavelength range of the spectrophotometer. A hypsochromic effect was observed shifting the wavelength from 445 to 380 nm. The EE was therefore determined by Eq. (1) since the volume of liposomes was negligibly compared to solvent volume.

$$EE (\%) = \frac{[\text{Lutein Total}] - [\text{Lutein Free}]}{[\text{Lutein Total}]} \times 100 \quad (1)$$

Absorbance measurements were carried out five times for each sample. Associated error (I) to EE determination is calculated according to the law of propagation of uncertainties (“Guide to the Expression of Uncertainty in Measurement. Geneva, Switzerland: ISO,” 2008) by Eq. (2).

$$I = 2\sqrt{\left(\frac{\partial EE}{\partial [\text{Lut. Tot.}]}\right)^2 \times U_{[\text{Lut. Tot.}]}^2 + \left(\frac{\partial EE}{\partial [\text{Lut. Free}]}\right)^2 \times U_{[\text{Lut. Free}]}^2} \quad (2)$$

where  $U$  is defined by Eq. (3) with SD as the SD and  $n$  as the number of repetitions of the measure.

$$U = \frac{SD}{\sqrt{n}} \quad (3)$$

## 3. Results and Discussion

### 3.1. Process Repeatability

In order to enable interpretations of the results obtained during this work (mean size, PDI, and EE), repeatability tests were first conducted. The repeatability was verified by conducting three experiments (Lut 01, Lut 01bis, and Lut 01ter) at 10 MPa, 313 K and a drug to lipid ratio of 1% w/w. The size distributions obtained are shown in **Figure 3** and demonstrate that this process has an excellent repeatability. The comparison of particle size distribution in terms of MD and SD (**Table 2**) evidences the high repeatability of the process. Indeed, the implemented process allowed, in a repeatable manner, the elaboration of liposomes with MDs of about 70 nm under the indicated conditions (Lut1).

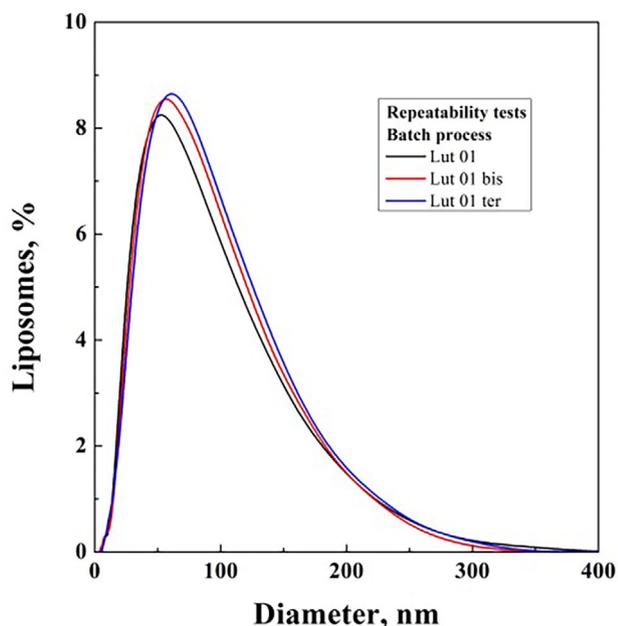


Figure 3. Size distribution obtained in repeatability tests.

Lutein EE, reported in Table 2, was also determined for 3 repeatability tests. For both processes, the produced liposomes encapsulated lutein in a repeatable manner with high encapsulation rates. At the chosen operating conditions for repeatability, the liposomes formed by the batch process had an average EE of  $85.2 \pm 1.2\%$ . Liposome production with supercritical  $\text{CO}_2$  allowed to entrap lutein with excellent encapsulation rates (above 82%).

### 3.2. Experimental Study Results

All the results obtained (mean size, particle size distribution, and EE), following the experimental design described in Table 1 and the analysis methods indicated in Section 2 (Experimental Section), are summarized in Table 3.

The encapsulation of lutein in liposomes by supercritical processes showed high EEs. Indeed, the EEs, in all operating conditions, were higher than 82% and can reach 91.9% for a pressure

of 10 MPa, a temperature of 308 K, and a drug to lipid ratio of 0.5% w/w. The influence of operating conditions on the efficiency of lutein encapsulation is described in the sections below.

### 3.3. Influence of Operating Conditions

#### 3.3.1. Influence of temperature

The influence of temperature on mean particle size and lutein encapsulation on produced liposomes was studied at 308, 313, and 318 K. These temperatures were chosen to avoid lutein degradation, and considering that the critical temperature for carbon dioxide is 304 K. Indeed, lutein degradation is accelerated at high temperatures,<sup>[40]</sup> especially above 333 K.<sup>[41]</sup>

The results are shown in Table 3. As can be seen in Figure 4, temperature does not have a significant impact on liposome size. For tests conducted under similar conditions (10 MPa and a drug to lipid ratio of 1% w/w) but at temperatures of 308 K (Lut 2), 313 K (Lut1), and 318 K (Lut3) no significant differences were observed in the size of the liposomes formed. Liposomes formed at 308, 313, and 318 K have diameters of  $73 \pm 39$  nm (Lut2),  $67 \pm 36$  nm (Lut1), and  $68 \pm 35$  nm (Lut3) respectively. In the temperature range studied (between 308 and 318 K) the increase in temperature resulted in a non-significant variation of vesicle diameter. The same type of observation is reported in the work of Zhao et al.<sup>[38]</sup> However, according to this mentioned work<sup>[38]</sup> a temperature increase can affect liposome size when temperature rises above 323 K.

The results shown in Table 3 report the lutein EEs in liposomes. The implemented process leads to a maximum lutein EE of  $91.9 \pm 2.9\%$  (Lut6) and  $90.6 \pm 1.6\%$  (Lut8) as optimal EE. In view of the EEs obtained for the different conditions, temperature does not seem to have a great impact on the EE of lutein inside liposomes. Looking to the literature, an effect of temperature variation upon the EE of lutein in liposomes formed by supercritical process has been observed by Xia et al.<sup>[37]</sup> According to the results published by Xia et al.<sup>[37]</sup> as well as that of Xu et al.,<sup>[42]</sup> this phenomenon can be explained by the fact that supercritical  $\text{CO}_2$  has a higher antisolvent power at higher temperatures. Indeed, the mixture between the phospholipid solution and  $\text{CO}_2$  leading to a single phase is easier when the inlet temperature is low.<sup>[42]</sup>

Table 3. Size, size distribution, and EE for the experimental design performed for the encapsulation of lutein.

Name test	Pressure [MPa]	Temperature [K]	Drug to lipid ratio[% w/w]	Liposome MD $\pm$ SD[nm]	EE $\pm$ SD[%]
Lut1	10	313	1	67 $\pm$ 36	83.8 $\pm$ 1.1
Lut2	10	308	1	73 $\pm$ 39	86.0 $\pm$ 1.1
Lut3	10	318	1	68 $\pm$ 35	89.3 $\pm$ 1.6
Lut4	15	308	1	68 $\pm$ 36	82.1 $\pm$ 3.7
Lut5	15	318	1	65 $\pm$ 35	84.9 $\pm$ 0.3
Lut6	10	308	0.5	77 $\pm$ 40	91.9 $\pm$ 2.9
Lut7	10	318	0.5	65 $\pm$ 33	84.0 $\pm$ 0.9
Lut8	15	308	0.5	75 $\pm$ 41	90.6 $\pm$ 1.6
Lut9	15	318	0.5	72 $\pm$ 39	89.6 $\pm$ 1.8

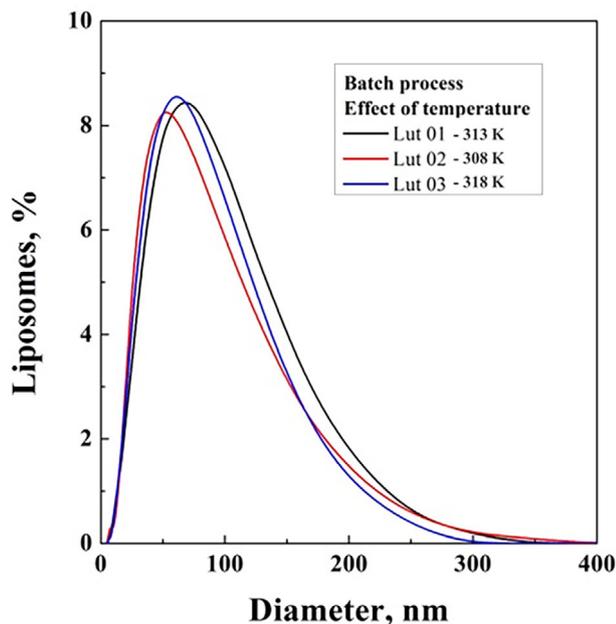


Figure 4. Influence of temperature on liposome size and size distribution for a pressure of 10 MPa and a drug to lipid ratio of 1% mass, produced.

The ability to form liposome/lutein complexes with high encapsulation rates (higher than 90%) using low temperature processes is interesting for several reasons. First of all, the advantage of these liposome elaboration processes in a supercritical medium at low temperatures allows to encapsulate lutein without any risk of thermal degradation. Moreover, operating at temperatures between 308 and 318 K limits energy costs compared to conventional liposome manufacturing processes operating at higher temperatures to allow the evaporation of the solvent. Finally, selecting a working temperature of 308 K makes it possible to optimize the energy costs associated with the preparation of liposomes.

### 3.3.2. Influence of Lutein to Lipid Ratio

The results summarized in Table 3 show that the lutein to lipid ratio does not seem to have an impact on the particle size distribution. Liposomes produced at 10 MPa and 318 K were compared, changing the DLR from 0.5% to 1% w/w.

The mean particle size did not vary significantly with variation in the lutein to lipid ratio. It varied from  $65 \pm 33$  nm at 0.5% w/w DLR to  $68 \pm 35$  nm at 1% w/w DLR. SDs were practically the same, i.e., the PDI of these particle size distributions were not affected by lutein to lipid ratio variation. However, Figure 5 shows that the particle diameter was systematically lower than 100 nm. The same trend was reported in the work of Zhao et al.<sup>[38]</sup>

Similarly, the lutein to lipid ratio does not seem to have an impact on lutein EE. There is probably no correlation between liposome size, EE, and lutein to lipid ratio in these studied conditions.

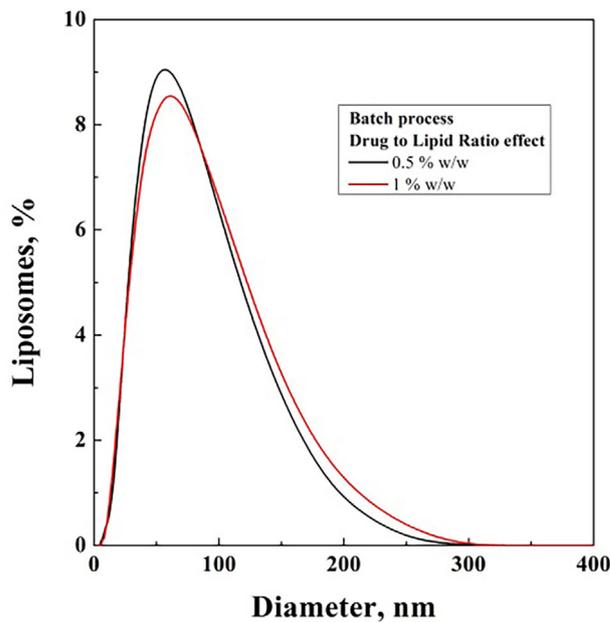


Figure 5. Influence of lutein to lipid ratio on liposome size distribution for a pressure of 10 MPa and a temperature of 45 °C, produced.

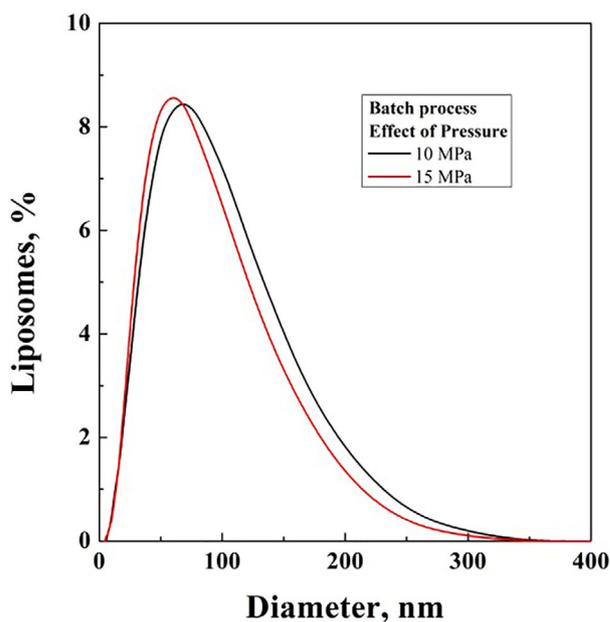


Figure 6. Influence of operative pressure on liposome size distribution for a drug to lipid ratio of 1% w/w and a temperature of 35 °C, produced.

### 3.3.3. Influence of Pressure

The results presented in Table 3 seem to show that the pressure does not significantly impact the particle size, the particle size distribution, and the lutein EE in the pressure range of 10–15 MPa. As no significant differences were observed in experiments conducted at 10 and 15 MPa (Figure 6), it seems likely that the CO<sub>2</sub>/lipid ratio obtained at 10 MPa is sufficient to incorporate lutein into the CO<sub>2</sub>/water emulsion in the autoclave. Once

the lutein is present in the emulsion, the phospholipids arrange themselves around the emulsion to encapsulate it to form the liposomes during the depressurization. The work of Xia et al.<sup>[37]</sup> led to the same type of observations concerning the influence of pressure on the EE of lutein even if the authors mention that an increase in pressure should lead to an increase in EE due to the increased solubility of CO<sub>2</sub> in the solvent. However, as in this study, Xia et al.<sup>[37]</sup> did not observe an increase in EE with increasing pressure from 8 to 16 MPa due to a bad mixing between ethanol and CO<sub>2</sub>. The lutein remains solubilized in the organic phase and is not encapsulated by the liposomes formed during depressurization.

However, it is necessary to perform studies at pressures above 30 MPa to confirm these trends and verify the effect of CO<sub>2</sub> amount on formed liposome particle size.<sup>[38]</sup> According to the results presented in this study, it is preferable to work at a pressure of 10 MPa in order to reduce the operation cost.

#### 4. Conclusions

A process for the development of lutein-loaded liposomes using supercritical CO<sub>2</sub> was developed. This batch process with a constant depressurization drop allows obtaining liposomes with minimal diameters of 65 ± 33 nm for a lutein EE of 91.9 ± 2.9%. This batch liposome production process makes it possible to produce vesicles with diameters among 30 and 110 nm. Temperature, pressure, and lutein to lipid ratio seem to have no impact on the liposome properties in the studied range (308–318 K; 10–15 MPa, and 0.5–1 wt%, respectively). Liposomal suspensions formed contain ethanol. The ethanol present in the solutions can easily be removed a simple solvent evaporation without significant degradation of the liposomes present in solution. The process proposed in this study is innovative compared to conventional liposome formulation processes due to the size of the liposomes obtained (less than 100 nm) and the excellent control that it offers. It represents a great potential for drug encapsulation by liposomes, particularly in nanomedicine. According to the work of Akinc et al.,<sup>[43]</sup> liposomes with these dimensions can be used as an encapsulation agent in nanomedicine, particularly in gene therapy. These types of liposomes allow drug delivery directly into cells by fusion with the cell membrane. Therefore, this study demonstrates that the use of this supercritical batch process is effective in forming nanoliposomes that can be used in nanomedicine.

#### Conflict of Interest

The authors declare no conflict of interest.

#### Keywords

human delivery, liposomes, lutein, process optimization, supercritical fluids

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