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Research paper

Use of supercritical CO_2 for the sterilization of liposomes: Study of the influence of sterilization conditions on the chemical and physical stability of phospholipids and liposomes

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ABSTRACT

The effects of four potential supercritical carbon dioxide ($ScCO_2$) sterilization conditions on the chemical stability of 9 phospholipids and on the physicochemical characteristics of liposomes consisting of stable phospholipids, as well as their sterilization efficiency were evaluated. These conditions were : C1 ($ScCO_2$/70 °C/150 bar/240 min), C2 ($ScCO_2$/0.25 % water/ 0.15% H_2O_2/ 0.5% acetic anhydride/38° C/85 bar/45 min), C3 ($ScCO_2$/0.08 % peracetic acid/35° C/104 bar/180 min) and C4 ($ScCO_2$/200 ppm H_2O_2/40 °C/270 bar/90 min). The results showed for phospholipids, a significant increase in hydrolysis products of 3.77 to 14.50 % and an increase in oxidation index of 6.10 to 430.50 % with unsaturated phospholipids for all tested conditions while with saturated phospholipids, no significant degradation was observed. Concerning the liposome formulation, no change in dispersion color and no phospholipid degradation were observed. However, a decrease in liposome size from 126.90 nm to 111.80 nm, 96.27 nm, 99.60 nm and 109.13 nm and an increase in the PdI from 0.208 to 0.271, 0.233, 0.285, and 0.298 were found with conditions C1, C2, C3 and C4 respectively. For the sterilization efficiency, conditions C1, C2 and C3 achieved the required sterility assurance level (SAL) of 10^{-6} for liposomes.

1. Introduction

Liposomes are spherical vesicles composed of one or more lipid bilayers able to load drugs and deliver them efficiently to cells. They are generally formulated with phospholipids and sterols and may include lipids combined with hydrophilic polymer [1,2]. Liposomes are currently of great importance in the pharmaceutical and therapeutic fields due to their excellent biocompatibility, biodegradability, and

Abbreviations: CFU, Colony forming unit; CO_2, carbon dioxide; $CuSO_4$, copper (II) sulfate; DC-cholesterol, dimethylaminoethane-carbamoyl cholesterol hydrochloride; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DSPC, 1,2-distearoyl-*sn*-glycero-3-phosphocholine; DSPE-PEG_{2000}, distearoylphosphatidylethanolamine polyethylene glycol 2000; EPC, egg phosphatidylcholine; FFA, free fatty acid; H_2O_2, Hydrogen peroxide; HEPC, Hydrogenated Egg phosphaditylcholine; HEPES, 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid; HPTLC, high performance thin layer chromatography; HSPC, hydrogenated Soy phosphatidylcholine; LPC, 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphocholine; LPE, 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphoethanolamine; LPL, lysophospholipid; PAA, peracetic acid; pCO_2, carbon dioxide critical pressure; PdI, polydispersity index; SAL, sterility assurance level; $ScCO_2$, Supercritical Carbon Dioxide; SPC, Soy phosphatidylcholine; TCO_2, carbon dioxide critical temperature; TLC, thin layer chromatography; UV, ultraviolet; w/w, weight/weight.

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cellular affinity, as well as their ability to improve the solubility, bioavailability, and action of active compounds, ensure their controlled release and minimize their adverse effects. Indeed, many liposome formulations are commercially available and undergoing clinical trials, including for the treatment of cancers, infectious, cardiovascular and inflammatory disorders [1–5]. Since most liposomal formulations are intended for parenteral administration and sterility is mandatory for this administration route, sterilization is an important step in their production process. Sterilization is a validated process delivering a product free of viable microorganisms without altering its properties [6]. However, liposome sterilization remains a challenging task because liposomes are very sensitive and susceptible to physicochemical degradations. Indeed, conventional sterilization methods such as heat treatment, ultraviolet and gamma irradiation are inappropriate for liposomes sterilization because they induce physicochemical alterations such as phospholipid oxidation and hydrolysis, vesicle aggregation, phase transition, degradation, leakage and change in encapsulated drug release [7]. Ethylene oxide is also unsuitable because it is a flammable and explosive gas that can form toxic residues [7]. Thus, sterilizing filtration and aseptic manufacturing are the currently accepted methods for preparing sterile liposomes. However, these methods also have limitations. Sterilizing filtration is not applicable when the liposomes size is larger than 200 nm. Moreover, it is ineffective in removing viruses and bacteria smaller than 200 nm [7]. Aseptic manufacturing is quite complicated and very expensive. In addition, it is not possible to assess the sterility assurance level (SAL) for aseptic production. These limitations make the search for an alternative method for liposome sterilization necessary [7].

Supercritical Carbon Dioxide ($ScCO_2$) is a widely used fluid in the pharmaceutical field, especially as an eluent in chromatography or as a solvent in extraction and in synthesis processes. It is also used as solvent in the manufacture of solid forms and liposomes [8–13]. In addition to these applications, $ScCO_2$ has sterilizing properties and is now a promising strategy for the sterilization of sensitive products, including liposomes [7]. Compared to other sterilization agents, $ScCO_2$ is environmentally friendly, non-flammable, non-toxic, chemically non-reactive, physiologically safe, affordable, and cheap. In addition, its relatively low critical parameters ($TCO_2 = 31.1$ °C, $pCO_2 = 73.8$ bar) allow for low temperature sterilization, and thus the sterilization of temperature-sensitive products [7]. Given these advantages of $ScCO_2$, this technology could be a good alternative technique for liposome sterilization. To this end, two approaches can be considered. On the one hand, $ScCO_2$ is already used for liposome production, and its sterilizing power could be used for liposomes production and sterilization in a one-step approach. In this case, the operating conditions should allow to fabricate liposomes with acceptable characteristics while reaching the SAL required for pharmaceutical products. On the other hand, $ScCO_2$ could also be used for the sterilization of liposomes produced by other conventional methods. In this case, the operating conditions must maintain liposomes characteristics and achieve the SAL required for pharmaceutical products [7].

While many works on the use of the $ScCO_2$ technology for the sterilization of various pharmaceutical and biomedical products have been reported and some of them have been licensed, there are very few investigations on its applicability to liposome sterilization. In a previous study, we investigated for the first time the possibility of using $ScCO_2$ for the sterilization of liposomes after their production through the evaluation of the effects of 4 potential sterilization conditions identified in the literature, on the physicochemical characteristics of liposomes containing cholesterol, dimethylaminoethane-carbamoyl cholesterol hydrochloride (DC-cholesterol), egg phosphatidylcholine (EPC) and distearoylphosphatidylethanolamine polyethylene glycol 2000 (DSPE-PEG_{2000}) [14]. The results showed a color change of the liposome dispersion from white to yellow after treatment with the four sterilization conditions. Changes in liposome size, PdI and zeta potential were also observed with all 4 conditions. These changes in liposomes characteristics could reflect phospholipid degradation by hydrolysis and oxidation, the major chemical degradation pathways of phospholipids [14]. Indeed, a similar color change was reported by Kikuchi and colleagues with a liposome formulation containing EPC after autoclaving at 121 °C for 20 min and was attributed to oxidation of the olefin moieties of EPC by residual oxygen [15]. As a general trend, all components of liposome formulations that display high peroxide value due to the presence of olefinic moieties within their chemical structure are prone to oxidation in the presence of oxidants (e.g. hydrogen peroxide and peracetic acid) used as additives in the sterilization. Hydrolysis of the ester linkages found in phospholipids or in other liposome components is also known to cause the deterioration of the liposomes, i.e. by affecting their physical stability. Hydrolysis could also be facilitated by acidification of the sterilization medium caused by the dissolution of CO_2 within the water-based formulation and may also impact the stability of some drugs with ester linkages [7,14,16–23].

To our knowledge, the chemical stability of phospholipids under $ScCO_2$ sterilization conditions has never been investigated.

The aim of the present study was to evaluate the effects of four potential sterilization conditions on the chemical stability of phospholipids and possibly on the physicochemical characteristics of liposomes consisting of stable phospholipids, as well as the sterilizing efficiency of the tested conditions. It would allow to understand the physicochemical changes of the liposomes observed and to verify the sterilizing efficiency of the potential sterilization conditions tested in the previous study.

2. Materials and methods

2.1. Materials

EPC, Hydrogenated Egg phosphaditylcholine (HEPC), Soy phosphatidylcholine (SPC), hydrogenated Soy phosphatidylcholine (HSPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), DSPE-PEG2000, cholesterol, DC-cholesterol, 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (LPC), 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphoethanolamine (LPE) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Acetic anhydride, copper (II) sulfate anhydrous ($CuSO_4$), orthophosphoric acid 85 % were purchased from Merck (Germany). Hydrogen peroxide (H_2O_2), chloroform HPLC grade, triethylamine and 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES) were purchased from Sigma Aldrich, (Belgium). Methanol analytical grade, ethanol absolute analytical grade and peracetic acid (PAA), were purchased from Fisher scientific (Belgium). Spores suspension of *Bacillus atropheus* was purchased from Crosstex (USA). Tryptic soy broth and tryptic soy agar were purchased from Sigma Aldrich (Belgium). Ultrapure water was produced by a Milli-Q system (Millipore, Bredford, MA, USA). All other reagents and solvents were of analytical grade.

2.2. Phospholipids and liposome formulation

The chemical stability of 9 phospholipids was studied and a liposome formulation was selected based on the stability results of the phospholipids.

Phospholipids were dispersed individually in 10 mM HEPES Buffer pH 7.4 at a concentration of 5 mg/mL.

Liposomes were prepared by thin film hydration method. Briefly, the appropriate amount of lipids was dissolved in ethanol in a 100 mL round-bottomed flask. The ethanol was then removed by evaporation under reduced pressure with a rotavapor (Büchi® R-200, Sigma® Aldrich, Germany) at 30 °C for 1 h. The thin lipid film obtained was then hydrated with 4 mL of HEPES buffer pH 7.4 and the suspension was then extruded successively three times through the 0.4 μm polycarbonate filters and three times through the 0.2 μm filters (Whatman® Nuclepore Track-Etch Membrane) using a liposome extruder (LipexTM Extruder,

Northern Lipids Inc., Vancouver, Canada).

2.3. Phospholipids and liposomes treatment by $ScCO_2$

Phospholipids and liposome dispersions were subjected to four $ScCO_2$ potential sterilization conditions (Table 1).

The treatment was performed using a $ScCO_2$ apparatus consisting of a N27 carbon dioxide (CO_2) bottle (Air liquide, Belgique), a pump (ISCO model 260 D), a 20 mL stainless steel autoclave (Autoclave® Engineering, France), a heating and stirring plate equipped with a temperature controller (Heidolph MR 3001 K, Allemagne), a manometer (WIKA, Belgique), CO_2 inlet and depressurization valves. Two (2) mL of phospholipids or liposome dispersion were introduced directly into the autoclave with or without additives, and then the whole system was thermostatized to the required processing temperature according to the tested condition, in the oil bath for 5 min. The system was then pressurized with CO_2 at the required pressure according to the tested condition, using the pump connected to the CO_2 bottle. After the required treatment time (depending on the condition), the $ScCO_2$ was removed by slow depressurization and the content was recovered for the various characterization tests.

2.4. Chemical stability of individual phospholipids and liposomes

2.4.1. Determination of lysophosphatidylcholine (LPC) or lysophosphatidylethanolamine (LPE) contents

The determination of the LPC and LPE contents, the main hydrolysis products of phosphatidylcholine and phosphatidylethanolamine respectively was performed by a validated high performance thin layer chromatography (HPTLC) method according to that described by Saetern et *al.* [23]. Phospholipid or liposome dispersions and standards were dissolved in methanol and then automatically deposited on HPTLC plates (Merck HPTLC silica gel 60 F_{254}, 10 × 20 cm) using Automatic TLC Sampler 4 (CAMAG, Switzerland). The following constant deposition parameters were used: volume 10 µL, deposition rate 150 nL/s, strip length 5 mm, gap from the bottom edge of the plate 15 mm. After drying, the plates were developed in a twin trough chamber (10 × 20 cm Camag, Switzerland) containing 10 mL of mobile phase (Chloroform/Methanol/triethylamine/water (30:35:34:8, v/v)). Development was stopped when the solvent migrated 70 mm. Plates were dried at room temperature for 10 min and then post-chromatographic derivatizated by dipping 3 × 5 s into a 10% $CuSO_4$ solution acidified with 8 % of 85% phosphoric acid followed by heating at 170 °C for 30 min. Detection and quantification of LPC or LPE were based on reference substances and were performed by densitometry at 530 nm using the photodensitometer TLC scanner 4 (Camag, Switzerland). The parameters used for detection and quantification were: scanning speed 5 mm/s, slit dimension 3.00 mm × 0.45 mm (micro) and a deuterium-tungsten lamp. For the establishment of the calibration curve, four concentrations (5, 25, 50, 100 µg/mL) of LPC or LPE reference substance in methanol, included in the validation range were prepared. The LPC or LPE content was determined using the equation below:

$$\text{LPC or LPE content}\,(\%) = \frac{\text{LPC or LPE concentraction}\,(\mu g/mL)}{\text{Initial phospholipid concentration}\,(\mu g/mL)} \times 100$$

2.4.2. Determination of oxidation index (for unsaturated phospholipids)

The oxidation index was estimated by measuring the conjugated dienes by UV/visible spectrophotometry according to the method described by Qi et *al.* [28]. 100 µL of each sample was dissolved in 10 mL of ethanol. Samples were analyzed from 200 to 400 nm spectra with ethanol as a reference using spectrophotometer. Conjugated dienes, as the oxidation form of phospholipids reflecting the extent of lipid oxidation, have a maximum absorbance at 233 nm, so the oxidation index was calculated by the following equation:

$$\text{Oxidation index} = \frac{(A233 - A300)}{(A215 - A300)}$$

A233, A215 and A300 correspond to the absorbance at 233, 215 and 300 nm.

The absorbance at 300 nm was used to rectify the UV absorption baseline in the spectra.

2.5. Physicochemical characterization of liposomes

2.5.1. pH

The pH of liposome dispersion was measured using a pHmeter (Mettler Toledo®, UK).

2.5.2. Particle size, PDI and zeta potential

Z-average size (nm), PdI and zeta potential (mV) of liposomes were determined in ultrapure water by Dynamic Light Scattering at 25 °C using the Malvern Zetasizer® (Nano ZS, Malvern Instrument, UK).

2.6. Assessment of the sterilizing efficacy of the four $ScCO_2$ potential sterilization conditions: SAL determination

2 mL of liposome dispersion were contaminated under aseptic conditions with the spores of *Bacillus atropheus* at 1.15 x10^6 Colony forming unit (CFU)/mL using 100 µL of spore suspension (2.3 x10^6 spores/100 µL, Crosstex, USA) and then submitted to the 4 potential sterilization conditions. After the process, 1 mL and 1 µL of treated and untreated contaminated samples were plated on trypticase soy broth and trypticase soy agar (Sigma Aldrich, Belgium) and incubated at 37 °C for 7 days. Colonies were then counted, and the SAL was determined using the following equation:

$$\text{SAL} = \log\left(\frac{\text{number of CFU in untreated sample}}{\text{number of CFU in treated sample}}\right)$$

2.7. Statistical analysis

All experiments were carried out in triplicate (n = 3). All graphs and statistical analyses were performed using GraphPad Prism 5 for Windows version 5.03. One- way Anova with Dunett post-test or a non-parametric T-test were used for comparison. P values < 0.05 were considered statistically significant.

Table 1
Parameters of the four potential $ScCO_2$ sterilization conditions.

Condition	Additives*	Temperature (°C)	Pressure (bar)	Exposure time (min)	References
C1	–	70	150	240	[14,24]
C2	0.25 % H_2O /0.15 % H_2O_2 / 0.5% acetic anhydride	38	85	45	[14,25]
C3	0.08 % PAA	35	104	180	[14,26]
C4	200 ppm H_2O_2	40	270	90	[14,27]

* Additive amounts is based on the volume of autoclave.

3. Results and discussion

3.1. Effects of $ScCO_2$ sterilization conditions on the chemical stability of phospholipids

The chemical stability of phospholipids was investigated by the determination of LPC or LPE contents and the increase of the oxidation index before and after their exposition to the four potential sterilization conditions C1, C2, C3 and C4 (as described in Table 1). When exposed to excess moisture, heat, and light, phospholipids can undergo hydrolysis of their glycerol-fatty acids (FFA) ester bonds to form lysophospholipids (LPL), an intermediate degradation product that can further degrade into glycerophospholipids and a second FFA. Because the first hydrolysis step is fast, LPL accumulation is usually observed, which can therefore serve as an indicator of the overall hydrolysis process [29]. Phosphatidylcholine can be hydrolyzed to LPC and FFA, whereas LPE and FFA are formed by the hydrolysis of phosphatidylethanolamine. Phospholipids containing unsaturated fatty acids are known to undergo oxidative reactions under the influence of air or oxygen, light, heat, metal ions and acidic or oxidizing agents [30]. Since the oxidation of phospholipids is a complex phenomenon leading to the formation of several intermediates, the determination of the oxidation index, based on the measurement of conjugated dienes which are forms of phospholipids oxidation reflecting the degree of lipid oxidation, is often used [28]. The results showed a significant increase in LPC content with EPC and SPC and a significant increase in oxidation index with all unsaturated phospholipids (EPC, SPC, DOPC, and DOPE) after treatment with all tested conditions (Table 2). The degradation of these phospholipids could be favored by the presence of unsaturated bonds in their structure. Indeed, when comparing EPC and SPC which are natural phospholipid mixtures, the highest increase in LPC content (14.5 ± 0.75 %) and oxidation index (165.85 ± 2.43 %) was found with SPC which contains more unsaturated phospholipids than EPC. These phospholipid degradations could be related, for condition C1, to the relatively high conditions of temperature, pressure and long exposure time. For conditions C2, C3 and C4, the effects of acidic (peracetic acid, acetic anhydride) and oxidizing (H_2O_2, peracetic acid) additives could be an explanation [14,30]. This result confirms the involvement of EPC degradation in the changes of color and other characteristics observed with the liposome dispersion in our previous study [14]. Indeed, at low concentrations, LPC provide a liposome-stabilizing effect, due to their preferred location in the outer membrane of the liposome. In contrast, at high concentrations, LPC forms mixed micelles with phospholipids and can affect liposome stability, resulting in increased membrane fluidity and permeability, altered drug release, liposome fusion or lysis [23,28,29,31]. Phospholipid oxidation products can also cause permeability changes in the liposome bilayers and alter liposome shelf-life [30]. In addition, hydrolysis and oxidation products of liposome can induce toxic effects upon parenteral administration [23,28,29]. However, no significant degradation was found with HEPC, HSPC, DPPC, DSPC and DSPE-PEG_{2000} after treatment by all conditions tested. This could be explained by the fact that these are saturated phospholipids, which are chemically more stable. Therefore, liposomes made from saturated phospholipids could be sterilizable under the conditions tested. To verify this hypothesis, the effects of the same sterilization conditions on a liposome formulation were evaluated.

3.2. Effects of $ScCO_2$ sterilization conditions on physicochemical characteristics of liposomes

Based on the stability of phospholipids, the composition of the studied liposome formulation is shown in Table 3.

In terms of visual appearance, no color change of the liposome dispersion was observed after treatment with all sterilization conditions tested. This result is in agreement with that of Kikuchi's team [15] who noted a color change with EPC-liposome after autoclaving at 121 °C for 20 min, while no color change was observed with HEPC liposomes. Furthermore, this confirms the involvement of EPC degradation in the color change observed with the liposome dispersion in our previous study where we evaluated the effect of the same sterilization conditions on a liposome formulation differing from the present one by the presence of EPC instead of HEPC [14]. As noted above, the chemical stability of HEPC which is a saturated phospholipid could be an explanation.

The other physicochemical characteristics of liposome dispersion prepared from this formulation before and after $ScCO_2$ are presented in Fig. 1.

A decrease in the pH value was observed after treatment with all conditions (Fig. 1A). This decrease was slight, from 7.48 ± 0.01 to 6.52 ± 0.03 and 6.55 ± 0.03 for conditions C1 and C4 respectively, and could be related to an acidification of the liposome dispersion by carbonic acid generated by carbon dioxide in aqueous medium [2,32]. However, this

Table 3
Composition of liposome formulation.

Components	% (w/w)
Cholesterol	0.6
DC-cholesterol	29.4
HEPC	50.05
DSPE-PEG2000	19.95

Table 2
LPC or LPE contents and oxidation index increase of phospholipids before and after $ScCO_2$ treatment.

Phospholipids	LPC or LPE content (%)					Oxidation index increase (%)				
	untreated	After C1	After C2	After C3	After C4	untreated	After C1	After C2	After C3	After C4
EPC	<0.50	4.80 ± 0.18	6.61 ± 0.26	5.08 ± 0.28	3.77 ± 0.09	–	32.14 ± 1.70	6.10 ± 1.50	90.21 ± 5.20	31.60 ± 1.90
HEPC	<0.50	<0.50	<0.50	<0.50	<0.50	–	ND	ND	ND	ND
SPC	<0.50	14.5 ± 0.75	6.84 ± 0.12	7.97 ± 0.23	3.83 ± 0.12	–	107.31 ± 0.24	58.43 ± 2.43	156.09 ± 2.43	165.85 ± 2.43
HSPC	<0.50	<0.50	<0.50	<0.50	<0.50	–	ND	ND	ND	ND
DPPC	< 0.50	0.52 ± 0,18	0.81 ± 0,28	< 0.50	< 0.50	–	ND	ND	ND	ND
DSPC	<0.50	0.60 ± 0,15	2.03 ± 0.23	<0.50	<0.50	–	ND	ND	ND	ND
DOPC	< 0.50	0.64 ± 0,09	<0.50	<0.50	<0.50	–	19.27 ± 70	310.44 ± 4.70	179.72 ± 5.80	19.85 ± 2.90
DOPE	< 0.50	< 0.50	< 0.50	< 0.50	< 0.50	–	58.30 ± 3.70	430.50 ± 7.50	270.00 ± 4.20	86.20 ± 2.40
DSPE-PEG2000	< 0.50	< 0.50	< 0.50	< 0.50	< 0.50	–	ND	ND	ND	ND

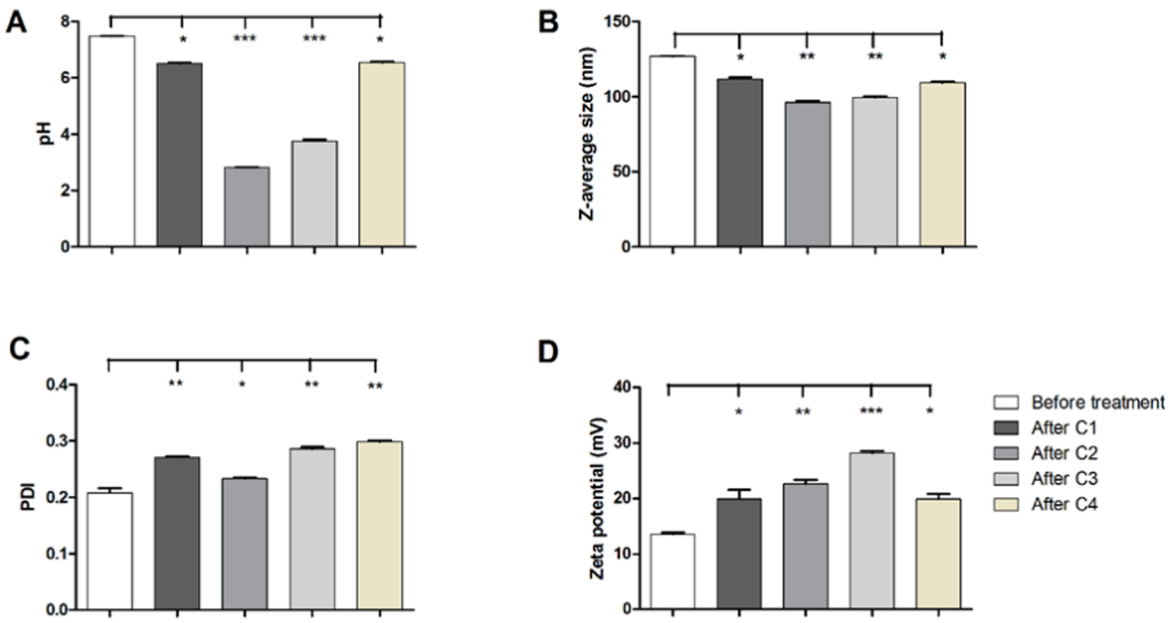

Fig. 1. Physicochemical characteristics of a liposome dispersion (HEPC/CHOL/DC-CHOL, DSPE-PEG_{2000}) before and after treatment with the four sterilization conditions. A: pH, B: Z-Average size (nm), C: PdI, D: Zeta potential (mV), (*): P values < 0.05, (**): P values < 0.01, (***):P values < 0.001.

decrease in pH was very significant, from 7.48 ± 0.01 to 2.83 ± 0.01 and 3.75 ± 0.09 for conditions C2 and C3 respectively and could be explained by the synergic effect of $ScCO_2$ and acidic additives used.

Concerning the liposome size and PdI (Fig. 1B and C), the results showed a decrease in liposome size, from 126.90 ± 0.52 nm to 111.80 ± 1.85 nm, 96.27 ± 1.96 nm, 99.60 ± 1.22 nm and 109.13 ± 1.35 nm after treatment with conditions C1, C2, C3 and C4 respectively, and an increase in the PdI from 0.208 ± 0.014 to 0.271 ± 0.002, 0.233 ± 0.003, 0.285 ± 0.006, 0.298 ± 0.003 for conditions C1, C2, C3 and C4 respectively. Being a formulation containing stable phospholipids, these changes could be attributed to reorganization of the phospholipids under the effect of $ScCO_2$. Indeed, when a liposome dispersion is subjected to $ScCO_2$, the mixture becomes an emulsion under pressure and the phospholipids rearrange. Upon depressurization, the CO_2 is removed, and the liposomes are reconstituted. Depending on the process conditions, the final characteristics of the liposomes can be the same or different from those of the initial liposomes [33].

In the case of zeta potential, an increase was found with all conditions tested (Fig. 1D). This increase was slight, from 13.56 ± 0.40 mV to 19.93 ± 2.87 and 19.93 ± 1.50 mV for conditions C1 and C4 respectively, and could be related to phospholipids rearrangement and pH change since zeta potential varies with pH. However, this increase in zeta potential was very significant, from 13.56 ± 0.40 mV to 22.60 ± 1.30 and 28.20 ± 0.65 mV for conditions C2 and C3 and could be explained by the synergic effect of $ScCO_2$ phospholipid rearrangement, injection of other positively charged ions and pH change by the additives.

Concerning LPC or LPE content, no significant increase was detected. This agrees with the results of the phospholipids chemical stability study which showed no degradation of HEPC and DSPE-PEG2000. This result could also be a confirmation of the chemical stability of saturated phospholipids and liposomes made of them.

However, it is important to note that despite the physical changes observed after $ScCO_2$ treatment, liposomes characteristics remain within acceptable limits for parenteral administration (size<200 nm and PdI<0.30, positive zeta potential).

3.3. *Sterilizing efficacy of the four $ScCO_2$ potential sterilization conditions*

In the pharmaceutical field, an effective sterilization process must achieve the required SAL of 10^{-6}, which is the probability that a product with an initial biological load (bioburden) of 10^6 colony-forming units of a given reference biological indicator is contaminated after the sterilization process [6,32]. The sterilizing efficacy of the conditions tested in this work was evaluated using *Bacillus atropheus* as a reference biological indicator. In the absence of a reference biological indicator for $ScCO_2$ sterilization, the latter, which is the reference biological indicator for ethylene oxide sterilization, is the most commonly used for the evaluation of the effectiveness of $ScCO_2$ sterilization due to the relatively similar nature of these two sterilizing agents [24–26]. The SAL obtained for liposome formulation with each of the four sterilization conditions is presented in the Table 4.

The results showed the achievement of a required SAL for pharmaceutical products ($\geq 10^{-6}$ or 6 log) with conditions C1, C2, and C3. These are in agreement with the SAL obtained by Karajanagi et *al.* [24], Bernhardt et *al.* [25], and Burns et *al.* [26] with C1, C2 and C3 respectively, using the same bioindicator. The mains mechanisms involved in the inactivation of bacterial spores by $ScCO_2$ are thought to be a structural modification of the inner membrane with increased permeability and alteration of the proteins involved in the spore germination [34–37]. Due to the high resistance of the spores to sterilizing agents, the use of $ScCO_2$ alone to achieve the SAL requires relatively high temperature and pressure conditions and a relatively long treatment time as in condition C1 (70° C, 150 bar for 4 h). Combining $ScCO_2$ with small amounts of additives such as H_2O_2, H_2O and acetic anhydride as in condition C2 (0.25 % water/ 0.15% H_2O_2)/ 0.5% acetic anhydride, 38° C, 85 bar for 45 min) and peracetic acid as in condition C3 (0.08 % peracetic acid, 35° C, 104 bar for 180 min) improves process performance and achieves sterility with milder conditions [2,6,7,32].

Table 4
Reduction of *Bacillus atropheus* spores contained in liposome dispersion after submission to the various sterilization conditions.

Conditions	Number of CFU/mL in untreated sample	Number of CFU/mL in treated sample	SAL (log reduction)
C1	1.2×10^6	0	> 6
C2	1.2×10^6	0	> 6
C3	1.2×10^6	0	> 6
C4	1.2×10^6	1.1×10^3	< 1

However, a SAL of less than 1 log was obtained for C4 condition in contrast to Donati et *al.* [27] who obtained a SAL > 6 log with the same condition. This result could be explained by the fact that they used a different bioindicator than ours (*Bacillus stearothermophilus* spores). Moreover, in their study, the spores were directly contacted with $ScCO_2$ in the reactor while in our work, the spores were incorporated into the liposome suspension before being subjected to the $ScCO_2$. The matrix effect through the formation of a protective corona around the spore could therefore also explain the low level of reduction we obtained compared to that obtained by Donati and colleagues.

Conditions C1, C2 and C3 achieved the SAL required for liposomes could therefore be used for their sterilization.

However, several considerations should be taken into account for the use of $ScCO_2$ as an innovative method for prefabricated liposome sterilization. Although the use of additives allows to obtain a good sterilizing efficiency in more convenient conditions of temperature, pressure and duration, their residual contents could pose safety of use problems and the conditions without additives are thus to be preferred [7]. Also, although transient, the acidification of the liposome suspension by $ScCO_2$, could be a constraint to its use as a sterilizing agent for liposomes containing pH-sensitive active ingredients or lipids. Their stability should be checked after processing [7]. Moreover, one of the mechanisms of microbicidal action of $ScCO_2$ is the increase of membrane permeability and fluidity and this could lead to a loss of encapsulated substances in liposomes, especially for hydrophilic ones. Their encapsulation efficiency should also be checked after the process [7].

4. Conclusion

This study showed that the $ScCO_2$ sterilization conditions tested affect the chemical stability of unsaturated phospholipids while saturated phospholipids appear to be stable under these conditions.

Although chemically stable, the physical characteristics of liposomes formed from saturated phospholipids are affected by the conditions tested. In general, the size of the liposomes decreases and the PdI increases after treatment with $ScCO_2$. These changes could be related to the reorganization of phospholipids in the $ScCO_2$ medium and the effect of additives. However, the observed variations remain within acceptable limits for pharmaceutical forms.

Conditions C1, C2 and C3 delivered the required SAL for liposomes. Considering the use of acidic and oxidizing additives in conditions C2 and C3 which significantly affect the pH of the liposome dispersion and whose residual contents could pose a problem of safety of use, condition C1 without additives would be the condition of choice.

We can conclude that condition C1 (70 °C, 150 bar, 240 min) would allow the sterilization of previously produced liposomes. In the case of liposomes loaded with active ingredient, it would be interesting to check the absence of leakage of the content after sterilization.

Considering that this reorganization of phospholipids leads to the formation of liposomes with acceptable characteristics for parenteral administration and based on previous studies performed in ours laboratory [12,38], the production and sterilization of liposomes made of saturated phospholipids, in a one step, could be considered.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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