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EFFECTS OF SUPERCRITICAL CARBON DIOXIDE UNDER CONDITIONS POTENTIALLY CONDUCIVE TO STERILIZATION ON PHYSICOCHEMICAL CHARACTERISTICS OF A LIPOSOME FORMULATION CONTAINING APIGENIN

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KEYWORDS: Supercritical carbon dioxide ; Liposomes ; Sterilization ; Physicochemical characteristics

ABSTRACT

In this work, the effects of four supercritical carbon dioxide ($ScCO_2$) conditions potentially conductive to sterilization, on physicochemical characteristics of a liposome formulation containing apigenin (AG) were investigated. 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 changes in color and a decrease in pH value of liposomes suspension after treatment with all tested conditions. Moreover, a decrease in liposome size with C1, C2 and C3 and an increase in the PDI with C1 and C3 were observed. For zeta potential, a decrease with C1, and an increase with C2 and C3 were found. Concerning the encapsulated AG, a moderate decrease, a strong decrease and a total disappearance were noted with conditions C1, C2 and C3 respectively.

HIGHLIGHTS

- Liposomes submitted to four ScCO2 conditions potentially conducive to sterilization
- Changes in color and pH value of liposomes observed after all ScCO2 processes
- Changes in liposome size and PDI observed after treatment by three conditions
- Changes in liposome surface charge observed after treatment by three conditions
- Decrease in apigenin content noted after liposome treatment by three conditions

GRAPHICAL ABSTRACT

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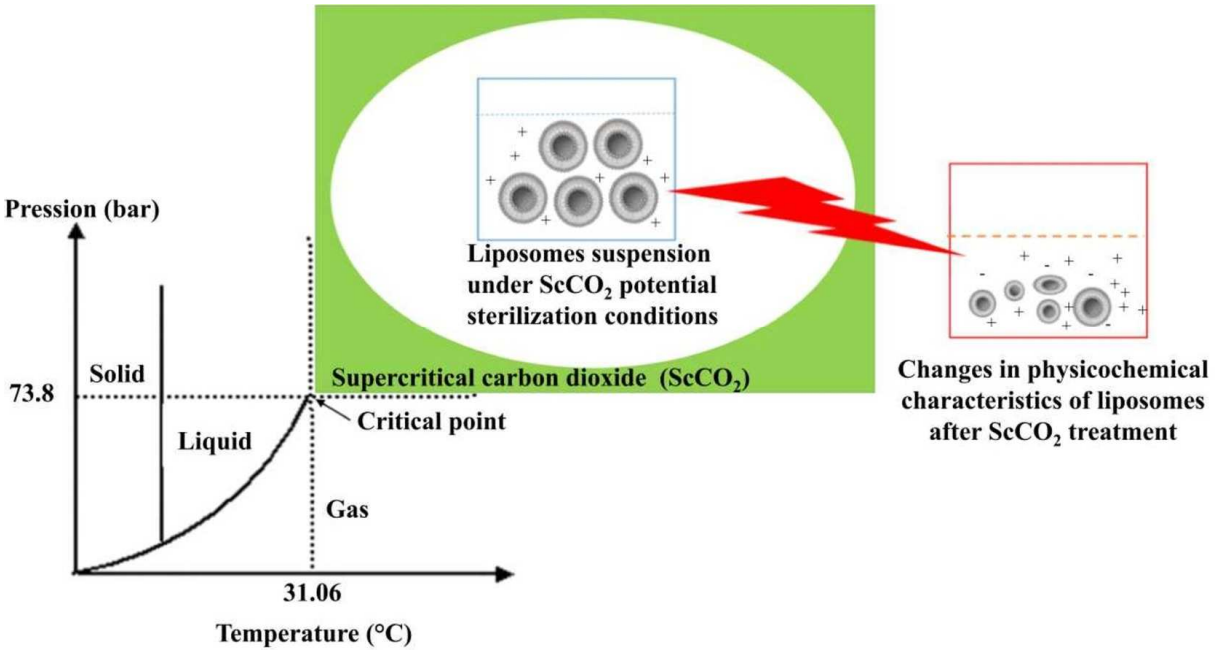

ABBREVIATIONS

AG, apigenin; AS, active substance; CO2,, carbon dioxide; DAOS, N-ethyl-N-(2-hydroxy-sulfopronyl) 3, 5-dimethoxyaniline; DC-cholesterol, dimethylaminoethane-carbamoyl cholesterol hydrochloride; DMSO, dimethylsulfoxide; DSPE-PEG2000, distearoylphosphatidylethanolamine polyethylene glycol 2000; EPC, egg phosphatidylcholine; H2O, water; H2O2, hydrogen peroxide; HEPES, acide 4-(2-hydroxy´ethyl) pip´erazine-1-ethane sulfonique; HPLC, high performance liquid chromatography; PAA, peracetic acid; PDI, polydispersity index; SAL, sterility assurance level; ScCO2, supercritical carbon dioxide.

1. Introduction

Liposomes are spherical vesicles constituted by one or more lipid bilayers and one or more aqueous compartments that can incorporate active substances (AS) either in an aqueous compartment (hydrophilic AS) or within the lipid bilayer (lipophilic AS) and deliver them to cells effectively [1], [2], [3]. Due to their high biocompatibility, biodegradability and good cellular affinity, as well as their ability to improve the solubility, the bioavailability and the action of active ingredients, to ensure their controlled release and to reduce their side effects, liposomes are nowadays of high interest in the pharmaceutical and therapeutic fields [2], [4], [5], [6]. Numerous liposome preparations are marketed or are the subject of clinical trials, mainly for the treatment of cancer, infectious, cardiovascular and inflammatory diseases [5], [6]. Sterilization represents a very essential step in the production of liposomes since most liposome formulations are intended for parenteral administration and sterility is a requirement in this case.

Sterilization can be defined as a validated process used to obtain a product free of viable microorganisms without altering the properties of sterilized product [7]. In the pharmaceutical field,

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a sterilization process can be considered valid if it achieves the required Sterility Assurance Level (SAL) of 10^{-6}. SAL is the probability that a product with an initial biological load of 10^6 colony-forming units of a given reference biological indicator (spores of one of the three bacterial species that are Geobacillus stearothermophilus, Bacillus pumilus, Bacillus atrophaeus) is contaminated after sterilization process. A product can therefore be labeled "sterile" if the theoretical probability that a viable microorganism is present is less than or equal 10^{-6} [7], [8].

However, due to the high sensitivity of liposomes and their predisposition to physicochemical alterations, liposome sterilization remains a problematic issue. Conventional sterilization methods such as heat (steam and dry air), chemicals (ethylene oxide), and gamma irradiation are unsuitable for liposomes sterilization because of physical and chemical degradations and toxicity issues of chemical methods. Heat sterilization is responsible for numerous physicochemical alterations of the liposomes such as aggregation, oxidation and hydrolysis of phospholipids, phase transition, degradation and leakage of encapsulated drug and change in drug release [2], [3], [9]. Sterilization by gamma irradiation also induces degradation of liposomes by lipid peroxidation, fragmentation and hydrolysis of phospholipids, formation of radical products, changes in pH and thermotropic behavior, alteration of drug release profile [1], [2], [9]. Ethylene oxide is a flammable and explosive gas that can form toxic, carcinogenic and mutagenic residues. Also, ethylene oxide sterilization is not applicable to liposomes in the form of an aqueous suspension [2], [9]. Filtration and aseptic manufacturing are recommended methods for the preparation of sterile liposomes. However, these accepted methods have also limitations. Filtration is relatively time-consuming, requires high pressure and aseptic, and is not applicable when the size of the liposomes is greater than 0.2 µm [2], [3], [9]. Moreover, filters with a pore diameter of 0.2 µm are ineffective in the retention of viruses and certain bacteria [2], [9]. Aseptic manufacturing is quite complex and complicated, in particular when several handling steps have to be carried out in sterile environments and very expensive [2], [9]. Also, the sterility assurance level cannot be evaluated for aseptic manufacturing in contrast to terminal sterilization, and there is still a potential possibility of contamination, in particular if the initial raw materials are not effectively sterilized [2]. In addition, certain methods such as sterilization by irradiation and ethylene oxide present environmental hazards due to the chemical or physical nature of the sterilizing agent. In view of the limitations of conventional methods for liposome sterilization, the search for an alternative green, effective and inexpensive method to ensure the sterility of liposomes without altering them is therefore imperative.

Supercritical carbon dioxide ($ScCO_2$) is a fluid combining the physicochemical properties of both liquid and gaseous carbon dioxide [10]. It is a fluid commonly used in the pharmaceutical field as solvent for extraction or organic synthesis and eluent in chromatography. It is also used as valuable solvent in manufacturing processes for solid forms such as micronisation, the production of nanospheres, solid dispersions, and porous polymeric structures containing various active substances. In addition, it is used in the production of liposomes [10], [11], [12], [13]. Moreover, $ScCO_2$ exhibits sterilizing properties and has now become a realistic approach to the sterilization of sensitive products. Indeed, several studies have demonstrated the bactericidal, virucidal, fungicidal and sporocidal properties of $ScCO_2$ [8], [9]. In addition, under appropriate operating conditions, $ScCO_2$ have proven its utility to sterilize sensitive biomedical and pharmaceutical products without

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any major change in their properties [8], [9], [10], [14], [15], [16], [17], [18], [19], [20], [21], [22]. Unlike other sterilizing agents, $ScCO_2$ is non-polluting, non-flammable, non-toxic, chemically inert, physiologically harmless, accessible and inexpensive. In addition, its relatively low critical parameters (T = 31.1 °C, p_{CO2} = 73.8 bar) allows for low-temperature sterilization, therefore to sterilize heat-sensitive products [2], [7], [8], [9], [10].

In view of the sterilizing power and the advantages of $ScCO_2$ in comparison to the conventional methods, and considering the fact that $ScCO_2$ is used in liposome manufacturing, this technology could be a viable alternative technique for liposome sterilization. Indeed, several authors have asserted that $ScCO_2$ can be used for producing and sterilizing liposomes in one single step, but this assertion has not been proven [2], [6], [11], [23], [24]. This approach is possible if the operating conditions allow to obtain liposomes with the desired characteristics and to obtain the required SAL (10^{-6}). $ScCO_2$ could also be used for the sterilization of liposomes after production. This second approach is possible if the operating conditions make it possible to maintain liposomes characteristics [9].

The aim of this work is to have a first overview on the opportunity to use $ScCO_2$ for the sterilization of liposomes through the evaluation of the effects of 4 potential sterilization conditions identified in the literature on their characteristics. This study was carried out using a liposome formulation previously validated in our laboratory [25]. This model formulation contains cholesterol, dimethylaminoethane-carbamoyl cholesterol hydrochloride (DC-cholesterol), egg phosphatidylcholine (EPC) and distearoylphosphatidylethanolamine polyethylene glycol 2000 ($DSPE\text{-}PEG_{2000}$), and apigenin (AG) as active drug. Physicochemical characteristics of empty or AG-encapsulated liposomes were benchmarked before and after $ScCO_2$ treatment.

2. Materials and methods

2.1. MATERIALS

DC-cholesterol, EPC and DSPE-PEG2000 were purchased from Avanti® Polar Lipid, Inc, (Alabaster, AL, USA), cholesterol was obtained from Sigma® Aldrich (Germany) and apigenin (AG) was supplied by Symrise Biotives® (Germany). Acide 4-(2-hydroxyéthyl) pipérazine-1-ethane sulfonique (HEPES) was purchased from Sigma® Aldrich (Allemagne), Merck® (Allemagne). Ultrapure water was produced by a Milli-Q system (Millipore, Bredford, MA, USA). All other reagents and solvents were of analytical grade.

2.2. LIPOSOME MANUFACTURING

The composition of the liposome formulation is shown in Table 1.

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Table 1
Composition of liposome formulation [25].

Components	%
Cholesterol	0.6
DC-cholesterol	29.4
EPC	50.05
DSPE-PEG2000	19.95
AG	5[a]

[a] molar percentage based on the total amount of lipid

Liposomes were prepared using the thin film hydration method. Briefly, the appropriate amount of lipids (and AG in the case of liposomes containing drug) were 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 3 mL of HEPES buffer pH 7.4 and incubated for 1 h at room temperature with magnetic stirring. 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). The next step was post-insertion pegylation which consisted of adding DSPE-PEG_{2000} prepared in HEPES buffer pH 7.4 to the extruded liposomes and then keeping the mixture in a water bath at 37 °C for 30 min under stirring. For liposomes containing AG, the last preparation step was the removal of the unencapsulated AG by dialysis through a cellulose acetate membrane (Spectra/Por® Dialysis Biotech CE Tubing MwCO membrane: 20 kD) at 4 °C in HEPES buffer pH 7.4 (20 mL buffer/mL liposomes) for 2 h under stirring. The HEPES buffer was renewed after 1 h. Liposome suspensions were stored at 4 °C until further testing. The final phospholipid concentration in liposome suspension was about 14 mM.

2.3. LIPOSOMES TREATMENT BY $SCCO_2$

Liposome suspensions were subjected to four $ScCO_2$ potential sterilization conditions selected from those identified in the literature as having achieved the sterility assurance level required for pharmaceutical and biomedical products (Table 2).

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Table 2
Potential $ScCO_2$ sterilization conditions.

Condition	Additives[a]	Temperature (°C)	Pressure (bar)	Exposure time (min)	Depressurization rate (bar/s)	Previous sterilized products	Bioindicators previously used	SAL previously obtained	Reference
C1	–	70	150	240	0.22	Polyethylene glycol (PEG) hydrogels	*Bacillus atrophaeus*	10^7	[16]
C2	0.25% H_2O /0.15% H_2O_2 / 0.5% acetic anhydride	38	85	45	0.22	Alginate/agarose hydrogel	*Geobacillus stearothermophilus, Bacillus atrophaeus, Bacillus pumilus*	$> 10^6$	[20]
C3	0.08% PAA	35	104	180	3.45	Methylprednisolone powder	*Geobacillus stearothermophilus, Bacillus atrophaeus*	$> 10^6$	[15]
C4	200 ppm H_2O_2	40	270	90	0.22	Bisphenol A glycidyl methacrylate, triethylene glycol dimethacrylate thermoset materials	*Geobacillus stearothermophilus*	$> 10^6$	[17]

[a] Additive amounts is based on the volume of autoclave

The treatment was performed using $ScCO_2$ apparatus consisting of a N27 carbon dioxide (CO_2) cylinder (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 pressure gauge (WIKA, Belgique), CO_2 inlet and depressurization valves. 2 mL of the liposome suspension (with or without AG) were introduced directly into the autoclave with or without additives and then the whole was thermostatized at the required treatment temperature according to the condition tested, in the oil bath for 5 min. The system was then pressurized with CO_2 at the required pressure according to the condition tested, using the pump connected to the CO_2 cylinder. After the required treatment time (depending on the condition), the $ScCO_2$ was removed by slow depressurization and liposomes were recovered for the various characterization tests. In addition to the untreated liposomes, other control tests were performed for each condition. For condition C1, a control in the absence of $ScCO_2$ (no $ScCO_2$-no additives) was performed. For conditions C2, C3, and C4 the following control tests were performed: (no $ScCO_2$-no additives), (no $ScCO_2$ +additives), ($ScCO_2$-no additives).

2.4. PHYSICOCHEMICAL CHARACTERIZATION OF LIPOSOMES

2.4.1. PH

The pH of liposome suspension was measured using a pHmeter (Mettler Toledo®).

2.4.2. 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).

2.4.3. PHOSPHOLIPIDS CONCENTRATION

The phospholipid (phosphatidycholine) concentration was assessed by an enzymatic method using N-ethyl-N-(2-hydroxy-sulfopronyl) 3, 5-dimethoxyaniline (DAOS) in a reaction producing a blue pigment. The absorbance of this pigment, which is proportional to the amount of phospholipids

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contained in a sample, was measured by UV–visible spectrophotometry. The assay was performed using a phospholipid assay kit supplied by Wako Pure Chemical Industrie, Ltd (LabAssayTM Phospholipid, choline oxidase-DAOS method). Briefly, 2 µL of liposomes and corresponding volumes of standard solutions were introduced into the wells of a 96-well microplate and 300 µL of reagent were added to each test sample and standard solutions. After shaking and incubating the plate at 37 °C for 5 min, absorbance was measured at 600 nm using a microplate reader (Thermo Scientific™ Multiskan™ FC Microplate Photometer). The phospholipid concentrations of the liposome samples were then determined from the equation of the standard calibration curve.

2.4.4. DETERMINATION OF ENCAPSULATED APIGENIN

The encapsulated AG was determined by high performance liquid chromatography (HPLC) according a method previously validated in our laboratory. Chromatographic analysis was performed using HPLC Agilent 1100 system. Altima C18 column (5 µm,4.6*250 mm, Grace Alltech) was used. The mobile phase was ultrapure water/acetonitrile (55:45, v/v) and the flow rate was 1 mL/min. The column temperature, injection volume and detection wavelength were 25 °C, 10 µL and 340 nm, respectively.

For the establishment of the calibration curve, five concentrations of AG (1, 5, 50, 100, and 200 µg/mL) were prepared according to the following procedure: 2 mg/mL stock solution of AG in dimethylsulfoxide (DMSO) was first prepared and then a 200 µg/mL stock solution in the mobile phase was prepared from the stock solution in DMSO. The other AG concentrations were then prepared by diluting the 200 µg/ mL solution in the mobile phase.

Before encapsulated AG determination, unencapsulated AG of both treated and untreated liposomes samples was removed by dialysis through a cellulose acetate membrane at 4 °C in HEPES buffer pH 7.4 for 2 h under stirring. For the preparation of the liposome samples, 100 µL of liposome suspension was dissolved in 900 µL of methanol to destroy the liposomes and release the encapsulated AG.

After analysis of the samples by HPLC, the concentration of encapsulated AG was determined from the equation of the calibration curve.

2.5. STATISTICAL ANALYSIS

All experiments were carried out in triplicate (n = 3). The results were expressed as mean ± standard error of mean (SEM). All graphs and statistical analyses were performed using GraphPad Prism 5 for Windows version 5.03. One- way Anova with Dunett post-test was used for comparison. P values < 0.05 (*), or < 0.01 (**), or < 0.001 (***) were considered statistically significant and P values ≥ 0.05 were considered statistically non-significant (ns).

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3. Results and discussion

3.1. EFFECTS ON PHYSICOCHEMICAL CHARACTERISTICS OF LIPOSOMES WITHOUT AG

The physicochemical characteristics of the liposomes without AG were determined before and after submission to the various potential sterilization conditions C1, C2, C3 and C4 (see Table 2).

In terms of visual appearance, a change in color of the liposome dispersion from white to yellow was observed after treatment with the four envisioned sterilization conditions. This color change could be explained by a degradation of phospholipids, in particular an oxidation of the EPC under the effect of the treatment conditions. Indeed, similar color change was reported by Kikuchi and coworkers with a liposome formulation containing EPC after autoclaving at 121 °C for 20 min and was attributed to an oxidation of the EPC. Also, they mentioned that the yellow or brown coloration of lecithins after heat treatment was due to the degradation products of unsaturated acyl chain of lecithins via peroxide [26]. EPC contains as fatty acid chains palmitic acid (16:0, 32%), palmitoleic acid (16:1, 1.5%), stearic acid (18:0, 16%), oleic acid (18: 1, 26%), linoleic acid (18:2, 13%), linolenic acid (18:3, less than 0.3%), arachidonic acid (20:4, 4.8%) and docosapentaenoic acid (22:5, 4%) [27]. Since it contains unsaturated fatty acids such as oleic acid, linoleic acid, arachidonic acid and docosapentaenoic acid, EPC is known to undergo oxidative reactions under the effect of air or oxygen, light, heat, metal ions and oxidizing agents. The reaction products can cause permeability changes in the liposome bilayers [27]. Cholesterol could also be subject to this oxidation reaction. For condition C1, the change in coloration could be related to the synergistic effect of temperature and $ScCO_2$ as this change was not observable after simple heating of liposomes in the absence of $ScCO_2$ (no $ScCO_2$-no additives). For the other conditions (C2, C3, C4), the phenomenon could be related to the synergic effect of $ScCO_2$ and the additives because no change in color was observed after liposomes treatment by these conditions in the absence of the additives ($ScCO_2$-no additives), in the absence of $ScCO_2$ (no $ScCO_2$ +additives), and in the simultaneous absence of $ScCO_2$ and additives (no $ScCO_2$-no additives). Indeed, H_2O_2 and peracetic acid (decomposed into acetic acid and H_2O_2) can act as sources of oxygen and thus promote the oxidation of phospholipids and cholesterol. A decrease in the pH value was also observed after treatment with all conditions. This decrease was slight, from 7.4 ± 0.02 to 6.8 ± 0.2 for conditions C1 and C4, and could be due to the effect of $ScCO_2$ since no change in pH was observed after liposomes treatment by these conditions in the absence of $ScCO_2$ (no $ScCO_2$ +additives) and in the simultaneous absence of $ScCO_2$ and additives (no $ScCO_2$-no additives), while after treatment in the absence of additives ($ScCO_2$-no additives), similar change in pH was found. The mechanism involved in this pH decrease could be an acidification of the liposome dispersion by carbonic acid generated by carbon dioxide in aqueous medium [2], [8]. However, this decrease in pH was very significant, from 7.4 ± 0.02 to 2.6 ± 0.1 and to 3.9 ± 0.2 for conditions C2 and C3 respectively. This could be explained by the synergic effect of $ScCO_2$ and additives since the same change in pH was noted after liposome treatment with these conditions in the absence of $ScCO_2$ (no $ScCO_2$ + additives) while after treatment in the absence of additives ($ScCO_2$-no additives) and in the simultaneous absence of $ScCO_2$ and additives (no $ScCO_2$-no additives), a

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slight decrease in pH and an absence of change were observed respectively. Acetic anhydride and peracetic acid respectively used as additives in conditions C2 and C3 have an acidic character and can decrease the pH of the liposome suspension. The other physicochemical characteristics studied for liposome dispersions without AG are presented in Fig. 1.

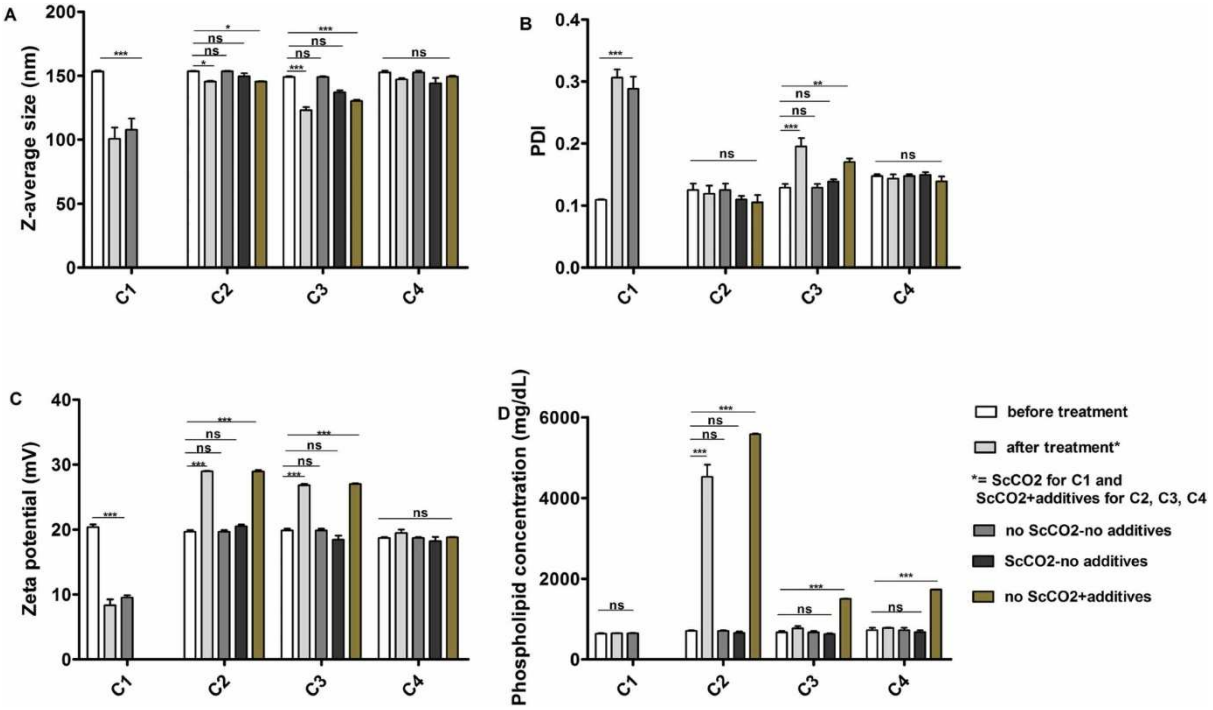

Fig. 1. Physicochemical characteristics of liposome dispersions without AG before and after submission to four potential sterilization conditions. A: Z-Average size (nm), B: PDI, C: Zeta potential (mV), D: Phospholipid concentration (mg/dL).

Concerning the liposome size and PDI (Fig. 1A and B), results showed a decrease in the liposome size, from 154 ± 1.3 nm to 100.6 ± 15.4 nm, 145 ± 1 nm and 123 ± 4 nm after treatment with conditions C1, C2 and C3 respectively and an increase in the PDI from 0.112 ± 0.005 to 0.306 ± 0.02 and to 0.195 ± 0.02 after treatment with conditions C1 and C3 respectively. These changes could be attributed to the rearrangement of phospholipids in the $ScCO_2$ medium. Indeed, when liposomal suspension is put into contact with $ScCO_2$, the mixture forms an emulsion under pressure. Phospholipids reorganize so as to form an emulsion which characteristics depend on the global composition (CO_2-water-phospholipid-active compound…). During the depressurization, CO_2 is released and liposomes reform. Depending on the operating conditions, the characteristics of the final liposomes can have similar or different characteristics than the ones of the initial suspension. Lipid degradation by oxidation and/or hydrolysis with change in bilayer properties (fluidity, permeability) could also be an explanation. For C1, the changes in size and PDI could be mainly related to the lipid degradation under the effect of temperature since the same variations were observed after simple heating at the temperature and for the duration of C1 in the absence of $ScCO_2$ (no $ScCO_2$-no additives). For conditions C2 and C3, the changes in size and PDI could be mainly due to the lipid degradation under the effect of additives. Indeed, the same variations in size and PDI were noted after addition of the additives to the liposome suspension followed by treatment with

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conditions C2 and C3 in the absence of $ScCO_2$ (no $ScCO_2$ + additives) while after treatment in the absence of additives ($ScCO_2$-no additives) and in the simultaneous absence of $ScCO_2$ and additives (no $ScCO_2$-no additives), no change was observed.

In the case of zeta potential (Fig. 1C), a decrease from 20 ± 1 mV to 8.3 ± 1.5 mV was observed after submission of the liposomes to condition C1 while an increase from 20 ± 1 mV to 29 ± 0.15 and to 27 ± 0.4 was found with conditions C2 and C3. The decrease in surface charge after treatment with condition C1, also observed after simple treatment of the liposomes in the absence of $ScCO_2$ (no $ScCO_2$-no additives) could, as for the variations in size and PDI, be mainly related to the thermal degradation of phospholipids, with the generation of compounds with different charge. For conditions C2 and C3, the increase in charge was also observed after adding additives to the liposome suspension followed by treatment in the absence of $ScCO_2$ (no $ScCO_2$ + additives), whereas after treatment in the absence of additives ($ScCO_2$- no additives) and in the simultaneous absence of $ScCO_2$ and additives (no $ScCO_2$-no additives), this variation was not evidenced. It could therefore be associated with the injection of other positively charged electrolytes by the additives in the liposome dispersion or with the interaction between the additives and the phospholipids resulting in the generation of other positively charged compounds. This increase in zeta potential could also result from the decrease in pH caused by acid additives since zeta potential varies with pH. However, it is important to note that for conditions C1, C2 and C3, the size and PDI of the liposomes remain within acceptable limits (size less than 200 nm and PDI less than 0.3) and the zeta potential stays positive.

In terms of phospholipid concentration (Fig. 1D), an apparent increase was noted after treatment of liposomes with condition C2. This could be a result of interference between one of the additives used (hydrogen peroxide) and the assay method. Indeed, the principle of the assay involves the production of hydrogen peroxide which reacts with other substrates to form a blue pigment whose intensity of coloration is proportional to the amount of phospholipids. The hydrogen peroxide already present in the liposome suspension could therefore cause an overestimation of the phospholipid concentration. This increase also observed with controls in the absence of $ScCO_2$ (no $ScCO_2$ +additives) for C2, C3 and C4 confirms this hypothesis and suggests that the added hydrogen peroxide was totally decomposed during the treatment with conditions C3 and C4 since the apparent increase was not observed after treatment for these two conditions. The phospholipid concentration, on the contrary, remained unchanged after treatment with conditions C1, C3 and C4. However, this does not exclude degradation (oxidation/hydrolysis) of the phospholipids. Indeed, the assay method used is based on the quantification of the choline part of phospholipids after enzymatic hydrolysis. In addition, the phenomenon of oxidation generally concerns the fatty acid chains of phospholipids. The assay method therefore does not only measure undegraded phospholipids and does not detect a decrease in phospholipid concentration by oxidation and/or hydrolysis.

No significant changes in size, PDI and zeta potential were detected after treatment of liposomes with condition C4. This could be explained by the rather gentle treatment parameters (40 °C for 90 min), the low amount of additive (H_2O_2) and its rapid decomposition into water. This condition

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therefore seems to be the most suitable compared to the other conditions to preserve the physicochemical characteristics of liposome formulation without active ingredient studied.

3.2. EFFECTS ON PHYSICOCHEMICAL CHARACTERISTICS OF LIPOSOMES CONTAINING AG

For liposomes containing AG, a change in color from white to pink was observed after treatment with all four conditions. This color change was not found with non-AG-containing liposomes and could therefore be attributed to a reaction involving AG. For condition C1, the change in coloration could be related to the synergistic effect of temperature and $ScCO_2$ as this change was not observable after simple heating of liposomes in the absence of $ScCO_2$ (no $ScCO_2$-no additives). For the condition C2, the phenomenon could be related to the synergic effect of $ScCO_2$ and the additives because no change in color was observed after liposomes treatment by this conditions in the absence of the additives ($ScCO_2$-no additives), in the absence of $ScCO_2$ (no $ScCO_2$ +additives), and in the simultaneous absence of $ScCO_2$ and additives (no $ScCO_2$-no additives). For the conditions C3 and C4 the phenomenon could be related to the effect of $ScCO_2$ since the same change in color was observed after liposomes treatment by these conditions in the absence of the additives ($ScCO_2$-no additives), while in the absence of $ScCO_2$ (no $ScCO_2$ +additives) and in the simultaneous absence of $ScCO_2$ and additives (no $ScCO_2$-no additives), no color change was found.

Alike "empty" liposomes, the formulation loaded with AG followed the same trend in pH variations and could be related to the same factors already discussed above. The other physicochemical characteristics studied for AG-containing liposomes are shown in Fig. 2. For size, PDI, zeta potential and phospholipid concentration (Fig. 2A, B, C, and E) the variations were similar to those observed in the absence of AG and could also be attributed to the phenomena discussed above.

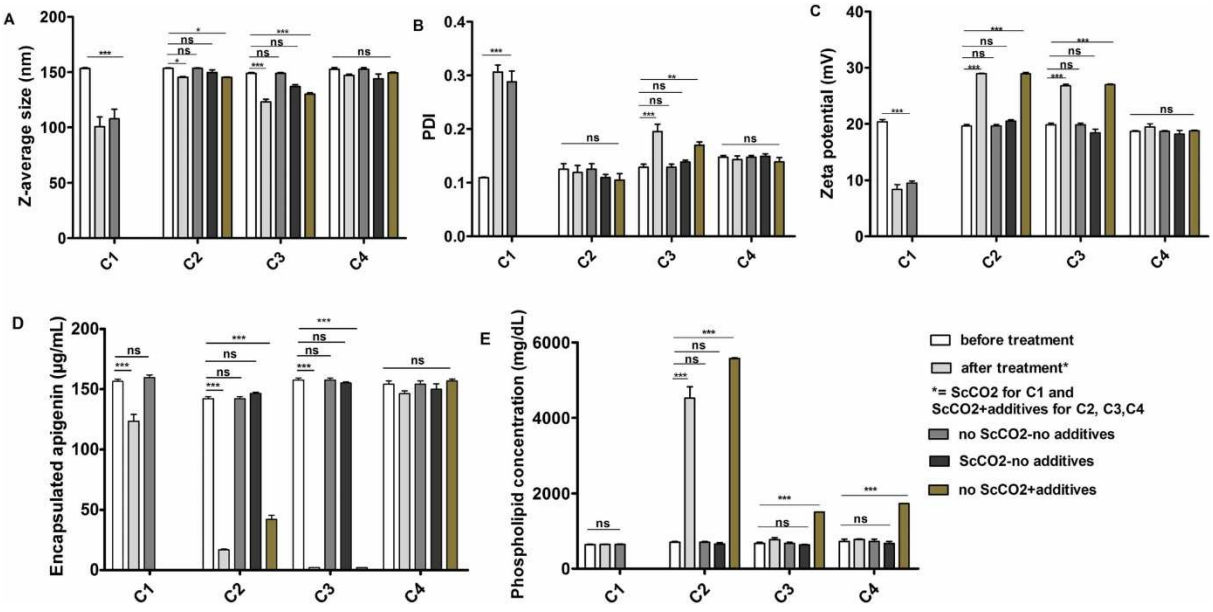

Fig. 2. Physicochemical characteristics of liposome dispersions with AG before and after submission to four potential sterilization conditions. A: Z-Average size (nm), B: PDI, C: Zeta potential (mV), D: Encapsulated apigenin (µg/mL), E: Phospholipid concentration (mg/dL).

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Concerning the concentration of encapsulated AG (Fig. 2D), a moderate decrease from 156 ± 1.4 µg/mL to 126 ± 6.3 µg/mL, a strong decrease from 156 ± 1.4 µg/mL to 17 ± 1 µg/mL, and a total disappearance of AG were noted after treatment of liposomes with conditions C1, C2 and C3 respectively. The decrease noted with condition C1 could be related to extraction and/or degradation of AG during treatment in the presence of $ScCO_2$ since this decrease was not observed after treatment in the absence of $ScCO_2$ (no $ScCO_2$-no additives). The strong decrease and the total disappearance of AG observed with C2 and C3 conditions respectively could be related to the effect of the additives, i.e. an acid degradation of AG. Indeed, the same variations in the amount of AG were observed after addition of the additives to the liposome suspension followed by treatment with conditions C2 and C3 in the absence of $ScCO_2$ (no $ScCO_2$ +additives) while after treatment in the absence of additives ($ScCO_2$-no additives) and in the simultaneous absence of $ScCO_2$ and additives (no $ScCO_2$-no additives), no changes were evidenced. As for size, PDI and zeta potential, no significant variation in the amount of encapsulated AG was noted after treatment of the liposomes with condition C4. This result confirm the fact that the C4 condition is the best compared to the other conditions in terms of maintaining the physicochemical characteristics of the liposome formulation used in this study.

4. Conclusion

Sterilization by $ScCO_2$ is probably a promising alternative to obtain sterility of liposomes if the operating conditions allow to maintain their characteristics. In this work, the effect of four potential sterilization conditions based on the use of $ScCO_2$ on the physicochemical characteristics of a liposome formulation was investigated. The results showed significant variations in characteristics after treatment with conditions 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) for both AG-free and AG-containing liposomes. However, for condition C4 ($ScCO_2$/ 200 ppm H_2O_2, 40 °C, 270 bar, 90 min), except a change in color, no significant variation was observed. In spite of changes observed with the liposome formulation under the potential sterilization conditions tested in this work, other conditions could make it possible to maintain the characteristics of the formulation studied. Also other liposome formulations may prove to be more stable after treatment under the conditions tested in this study.

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.

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