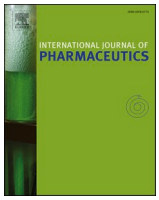
International Journal of Pharmaceutics 651 (2024) 123769

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm



Development and optimization of a one step process for the production and sterilization of liposomes using supercritical CO_2

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ARTICLE INFO

Keywords:
Liposome
Supercritical carbon dioxide
Green process
Quality by design
Production
Sterilization
One-step process
Encapsulation
Sterility assurance level

ABSTRACT

Liposomes are very interesting drug delivery systems for pharmaceutical and therapeutic purposes. However, liposome sterilization as well as their industrial manufacturing remain challenging. Supercritical carbon dioxide is an innovative technology that can potentially overcome these limitations. The aim of this study was to optimize a one-step process for producing and sterilizing liposomes using supercritical CO_2. For this purpose, a design of experiment was conducted. The analysis of the experimental design showed that the temperature is the most influential parameter to achieve the sterility assurance level (SAL) required for liposomes ($\leq 10^{-6}$). Optimal conditions (80 °C, 240 bar, 30 min) were identified to obtain the fixed critical quality attributes of liposomes. The conditions for preparing and sterilizing empty liposomes of various compositions, as well as liposomes containing the poorly water-soluble drug budesonide, were validated. The results indicate that the liposomes have appropriate physicochemical characteristics for drug delivery, with a size of 200 nm or less and a PdI of 0.35 or less. Additionally, all liposome formulations demonstrated the required SAL and sterility at concentrations of 5 and 45 mM, with high encapsulation efficiency.

1. Introduction

Today, liposomes have emerged as very interesting drug delivery systems for pharmaceutical and therapeutic purposes (Daraee et al., 2016). These nanovectors are increasingly used in the pharmaceutical field for the encapsulation of both hydrophilic and hydrophobic active pharmaceutical ingredients (APIs) such as small molecules but also for biopharmaceutical molecules or genetic materials (Daraee et al., 2016; Hasan et al., 2014, 2019; Dang et al., 2014; Lv et al., 2016). Due to their ability to enhance the solubility, bioavailability, and pharmacokinetics profile of active molecules, they improve the therapeutic index of drugs, thus increasing their efficacy while decreasing their side effects (Daraee et al., 2016; Dang et al., 2014). Given these merits, several liposomal drug products have been successfully approved by the United States Food and Drug Administration (FDA) and European Medicines Agency (EMA) and used in clinics over the last couple of decades. These included Doxil®/Caelyx® (Doxorubicin), DaunoXome® (Daunorubicin), AmBisome® (Amphotericin B), DepoCyt®/DepoCyte® (Cytarabine), Myocet® (Doxorubicin), Visudyne® (Verteporfin), DepoDur® (Morphine), Mepact® (MTP-PE), Exparel® (Bupivacaine), Marqibo® (Vincristine), Onivyde® (Irinotecan), Vyxeos® (Daunorubicin/cytarabine), Shingrix® (Recombinant varicella-zoster virus glycoprotein E), Arikayce® (Amikacine) (Liu et al., 2022). The therapeutic area mainly focuses on cancer therapy but also involves other areas, such as infection, anesthesia,

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https://doi.org/10.1016/j.ijpharm.2024.123769
Received 6 October 2023; Received in revised form 21 December 2023; Accepted 2 January 2024
Available online 4 January 2024

vaccine, lung disease, and photodynamic therapy (Liu et al., 2022). Most of liposomal formulations are administered parenterally making their sterility mandatory (Galante et al., 2016; Liu et al., 2022).

Despite the continued growth of the nanomedicine market, liposome-based nanomedicines still represent only a small fraction of the global pharmaceutical market, and their presence is much lower than other conventional pharmaceutical forms (Crommelin et al., 2020; Younis et al., 2022). In fact, the production of nanomedicines faces several challenges that limit their transfer from laboratory research and development to industrial production (Younis et al., 2022; Guimarães et al., 2021).

The main limitation for the commercialization of liposomes is the development of a suitable method for large-scale production and sterilization, also known as scale-up (Dymek and Sikora, 2022). Indeed, for liposomes to be used as an acceptable pharmaceutical product, their large-scale production must be reproducible, practically easy and economically feasible (Dymek and Sikora, 2022). Conventional liposome production and sterilization methods suffer from several shortcomings. Regarding their sterilization, conventional sterilization methods such as heat, ethylene oxide, ultraviolet and gamma irradiation are considered as unsuitable for liposome sterilization due to their sensitivity (Delma et al., 2021). Indeed, these methods increase the drug leakage, the hydrolysis and oxidation of phospholipids with the production of degradation products and the aggregation of vesicles with an increase in liposomes size and dispersity (Toh and Chiu, 2013; Delma et al., 2021). Although aseptic production and sterile filtration are recognized methods for producing sterile liposomes, they are not without limitations. Sterile filtration is not feasible when the liposome size is larger than 200 nm. It is also inefficient at removing bacteria smaller than 200 nm and viruses (Delma et al., 2021). Aseptic processing is relatively expensive and complex method, and it is impossible to estimate the SAL (Delma et al., 2021). Moreover, these sterilization methods are relatively time-consuming, require high energy and are thus expensive methods (Toh and Chiu, 2013). Regarding liposome production, the conventional methods are often multi-step processes, using a large amount of organic solvents with poor reproducibility and the need for various downstream steps making these processes easy to implement in the laboratory but rarely transferable to industrial scale under Good Manufacturing Practice (GMP) conditions (Karn et al., 2013; Maja et al., 2020; Bridson et al., 2006; Wagner and Vorauer-Uhl, 2011). The development of more innovative production technologies is therefore needed to ensure the translation of new nanomedicines from bench to bedside.

New innovative and/or sustainable technologies have emerged such as processes using supercritical fluids and microfluidics to overcome the problems encountered with conventional laboratory methods for liposome production (Maja et al., 2020). Although microfluidics has overcome some limitations by providing larger batch volumes, better reproducibility, and fewer steps, the use of large volumes of ethanol remains a limitation (Maja et al., 2020; Patil and Jadhav, 2014; Shah et al., 2019).

Today, supercritical carbon dioxide (SC CO_2) appears as an innovative technology that could overcome all these limitations. Indeed, SC CO_2 has a great microbicidal potential and represents an interesting alternative for the sterilization of sensitive products such as liposomes (Delma et al., 2021, 2022). The main mechanisms involved in the inactivation of bacterial spores by SC CO_2 are well established in the literature (Zhang et al., 2006, 2007; Rao et al., 2016; Setlow et al., 2016). Compared to other sterilization agents, SC CO_2 offers many advantages. It is, non-flammable, non-toxic, chemically inert, environmentally friendly, physiologically safe, inexpensive, and available. In addition, its critical parameters are low and achievable with simple equipment, allowing the sterilization of heat-sensitive products (Toh and Chiu, 2013; Reverchon et al., 2010; Ribeiro et al., 2020; Soares et al., 2019). Furthermore, this technology is now an innovative liposome production method that allows liposomes to be produced in a one-step process, eliminates or minimizes the need for organic solvents, and yields smaller, more densely distributed liposomes with improved encapsulation efficiency compared to conventional liposome production methods (Maja et al., 2020; Bigazzi et al., 2020; Penoy et al., 2021). Currently, there are several methods for the preparation of liposomes using SC CO_2 in different ways, which are described in our previous paper (Bigazzi et al., 2020). The Particles from Gas Saturated Solution (PGSS) process has already been used by Penoy et al. to prepare liposomes encapsulating hydrophobic and hydrophilic APIs in a single step, without the use of organic solvents and with a process that could be easily transferred to industrial scale (Penoy et al., 2022).

Taking advantage of the ability to produce liposomes with SC CO_2 and its sterilizing properties, one-step production and sterilization with SC CO_2 could be an innovative approach to the design of new liposomal drug delivery systems. Santos-Rosales and colleagues have already successfully used the SC CO_2 technology to produce and sterilize drug-loaded scaffolds in a single step process (Santos-Rosales et al., 2022). However, to our knowledge, the applicability of this approach to liposomes had never been demonstrated. This may be feasible if the processing conditions allow liposomes to be produced with the desired physicochemical properties required for pharmaceutical products. In the pharmaceutical field, a sterilization process is considered effective if it results in a SAL of 10^{-6}. In the context of biological validation of a sterilization process, this SAL is the probability that a material initially loaded with 10^6 colony-forming units of a specific biological reference will be contaminated after the process (Reverchon et al., 2010; Soares et al., 2019). In terms of size, it is important to use small vesicles (<200 nm) for parenteral delivery to avoid potential risks such as vesicle trapping and retention in smaller capillaries (Toh and Chiu, 2013). A PdI less than or equal to 0.30 is generally accepted (Danaei et al., 2018).

In our previous studies, three SC CO_2 conditions were identified that allowed to obtain liposomes required for SAL while maintaining the physicochemical properties for preformed liposomes (Bigazzi et al., 2020). In another study, two SC CO_2 conditions were identified that allowed the production of liposomes in a one-step process without the use of an organic solvent and with physicochemical properties compatible with drug delivery (Penoy et al.; 2021). Given the feasibility of achieving the required SAL and suitable liposome properties for parenteral administration based on our previous investigations, we hypothesized that the one-step production and sterilization of liposomes by SC CO_2 could be considered as an innovative process.

The aim of this study is thus to use a quality by design strategy, based on our previous studies, to find process parameters that allows the production of sterile liposomes with SC CO_2 in a one-step process.

2. Materials and methods

2.1. Materials

Soy phosphatidylcholine (SPC) (CAS number 97281-47-5), 1,2-dioleoyl-3-trimethylammonium-propane (chloride salt) (DOTAP) (CAS number 132172-61-3), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) (CAS number 4004-05-1), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethyleneglycol)-1000] (ammonium salt) (DSPE-PEG2000) (CAS number 474922-90-2), L-α-phosphatidylcholine hydrogenated (HSPC) (CAS number 97281-48-6), 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) (CAS number 816-94-4) and dimethylaminoethane-carbamoyl cholesterol hydrochloride (DC-CHOL) (CAS number 166023-21-8) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA), cholesterol (CHOL) was purchased from Sigma Aldrich (Belgium) (CAS number 57-88-5). Budesonide (CAS number 51333-22-3), Ph. Eur. 8.3 micronized, was obtained from Minakem (France), 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES) (CAS number 7365-45-9) ≥ 99.5% and sodium chloride (CAS number 7647-14-5) were purchased from Sigma Aldrich (Belgium). Ultrapure water was produced by a Milli-Q system

(Millipore, Bredford, MA, USA). Liquid CO_2 (CAS number 124–38-9) with a purity of 99.7% was purchased from Air Liquide (Belgium).".

Trypticase soy broth (TSB) (CAS number 105459), trypticase soy agar (TSA) (CAS number 105458), and fluid thioglycolate medium (FTM) (CAS number 108191) were purchased from Merck (Belgium). Sabouraud gentamicin chloramphenicol 2 agar (SGC2A) (CAS number 42037) was purchased from Biomerieux (France). Spore suspension of *Bacillus atrophaeus* (CAS number NC1338885) has been acquired from Crosstex (USA).

2.2. Liposome composition

4 different liposome formulations were used. Their composition is given in Table 1.

Formulation A was used for the experimental strategy and formulations B, C and D were used for the validation and transferability study.

2.3. Optimization using quality by design strategy (QbD)

A response surface A-optimal experimental design was constructed using JMP Pro 15 software (SAS Institute, Cary, USA) to test the main effects, two-factor interactions and quadratic effects of the selected continuous process parameters: pressure, temperature and contact time with SC CO_2. Based on the preliminary study and on the previous studies from Delma et al. (Delma et al., 2023) and Penoy et al. (Penoy et al., 2021), the supercritical process ranges parameters were determined: temperature ranging from 40 °C to 80 °C, pressure ranging from 120 bar to 240 bar and contact time from 30 min to 240 min were considered. The fixed parameters were stirring rate of 500 rpm and 2 mL of lipid dispersion with a lipid concentration of 5 mM. With regard to the critical quality attributes (CQAs) of liposomes, a size of less than 300 nm, a PdI of less than 0.35 and a SAL of 10^{-6} (log reduction > 6) were set. Three center points were added, and 3 runs were replicated leading to a total of 20 experiments distributed in blocks of two experiments to be run on the same day (Table 2). The optimization process was conducted by defining Z-average size (nm), PdI and SAL for each experiment of the experimental plan (Table 2). The results were analyzed with JMP Pro 15.

The response versus factor equation used are presented and described below:

Equation 1: equation for sterility

$$logit(y) = b_0 + b_1 T_p + e$$

Where y is the sterility; T_p is the temperature; b_i are the model coefficients; and e the random error.

Equation 2: equation for size and PDI.

$$y = (b_o + \gamma_{0k}) + b_1 T_p + b_2 P + b_3 T_c + b_{12} T_p \bullet P + b_{13} T_p \bullet T_c + b_{23} P \bullet T_c + b_{11} {T_p}^2 + b_{22} P^2 + b_{33} {T_c}^2 + e$$

Where y is the modelled response; T_p, P and T_c are the temperature, pressure and contact time respectively; b_i, b_{ij} and b_{ii} are the model coefficients; γ_{0k} is the random intercept for the k blocks; and e is the random error.

Table 1
Liposome composition.

Formulation	Composition	% (mol/mol)	Lipid concentration (mM)
A	SPC/CHOL/DSPE-PEG_{2000}	65/30/5	5 or 45
B	DOTAP/CHOL/DOPE	44.5/33.3/22.2	5 or 45
C	DSPC/CHOL/DSPE-PEG_{2000}	65/30/5	5 or 45
D	HSPC/CHOL/DSPE-PEG_{2000}	65/30/5	45

Table 2
A-Optimal experimental plan created with JMP Pro 15 program for sterilized liposomes with optimal physicochemical characteristics.

Experiment	Random block	Temperature (°C)	Pressure (bar)	Contact time (min)
1	1	60	180	240
2	1	80	120	30
3	2	40	240	240
4	2	40	120	135
5	3	60	180	135
6	3	80	240	240
7	4	80	180	135
8	4	60	240	30
9	5	60	120	30
10	5	60	120	30
11	6	60	180	135
12	6	40	180	30
13	7	80	120	240
14	7	80	120	240
15	8	40	240	135
16	8	80	180	30
17	9	40	120	240
18	9	40	120	240
19	10	80	240	30
20	10	80	180	135

2.4. Production and sterilization of liposomes

For each experiment, the appropriate amounts of lipids (Table 1) were pre-dispersed in 5 mL of HEPES buffer 10 mM, pH 7.4. 2 mL of the dispersion were then aseptically inoculated with *Bacillus atrophaeus* spores and submitted to the experiment condition using SC CO_2 apparatus consisting of a 20 mL stainless steel high-pressure cell (Autoclave® Engineering, France) (Fig. 1). Liposomes were then analyzed in terms of SAL as described in section 2.6.1 and size and PdI characterization as described in section 2.7.

2.5. Preparation of liposomes by the PGSS method

Liposomes were produced by a one-step PGSS method previously described in Penoy et al. (Penoy et al., 2021).

Lipids and APIs were pre-dispersed in HEPES buffer (65 °C, 1200 rpm and 15 min) and introduced into the high-pressure reactor. The reactor was heated at a specific temperature and CO_2 was pumped at the specific pressure regarding the optimized production and sterilization condition.

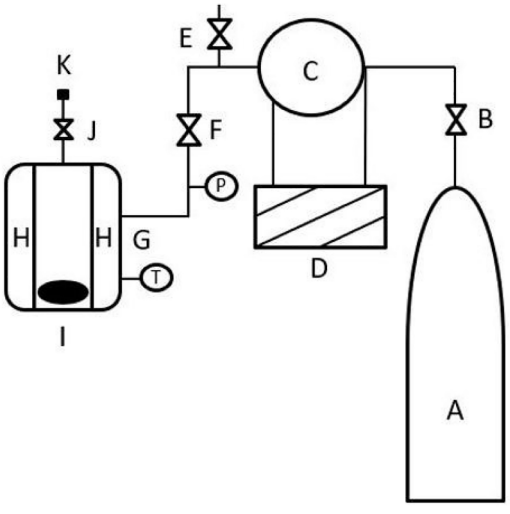

Fig. 1. Schematic representation of the high-pressure cell for sterilization assays. A: CO_2 bottle, B, E and F: ON/OFF valves, C: pump, D: refrigerant, G: high pressure reactor, H: heating jacket, I: stirrer, J: pneumatic valve, K: nozzle

The agitation rate was kept constant during all this study at 500 rpm. The supercritical content was then depressurized in the expansion tank.

2.6. Microbiological characterization of resulting liposomes

2.6.1. SAL determination

For each experiment the SAL was determined according to the method previously described by Delma et al. (Delma et al., 2023). Briefly, 2 mL of the lipid dispersion were contaminated under aseptic conditions with a specific concentration of $1.2x10^6$ CFU/mL of *Bacillus atrophaeus* spores and then submitted to the supercritical condition of the experiment. 1 µL and 100 µL of the lipid dispersion were inoculated under aseptic conditions on TSA and TSB culture mediums and incubated for 7 days at 35 °C before and after the supercritical treatment. After incubation time, the number of colonies were counted, and the SAL was determined following the equation 3 below:

Equation 3. SAL determination.

$$Log\left(\frac{NumberofCFUinuntreatedsample}{NumberofCFUintreatedsample}\right)$$

2.6.2. General sterility assessment

The sterility of the resulting liposomes was assessed by inoculating liposomes on different culture mediums (fluid thioglycolate medium (FTM), TSA, TSB and sabouraud gentamicin chloramphenicol 2 agar (SGC2A)) after the supercritical treatment of each experiment. The colonies were observed each day during 14 days of incubation to give slow-growing germs time to develop.

2.7. Particles size and size distribution characterization

Liposomes Z-average size (nm) and PdI were measured after each experiment by dynamic light scattering technique (DLS) using a Malvern Zetasizer® (Nano ZS, Malvern Instrument, UK) at 25 °C with a fixed angle of 90°. The samples produced were diluted in HEPES buffer (10 mM, pH value 7.4) to obtain a final concentration of 0.45 mM. All experiments were measured in triplicate (n = 3).

2.8. Budesonide encapsulation in liposomes

SPC or HSPC/CHOL/DSPE-PEG2000 (65/30/5 mol%) lipid dispersions (5 mM or 45 mM lipid concentration) in HEPES buffer containing 5 mol% budesonide were prepared and subjected to validated PGSS SC CO_2 conditions.

2.8.1. Liposomes purification

Liposome dispersions were purified by dialysis using Spectra/Por° Dialysis membrane in cellulose of 20 kD with a diameter of 10 mm purchased from VWR Chemical. The validated parameters used were: 3 h of dialysis with 20 mL of HEPES buffer for 1 mL of liposomes at 4 °C and 200 rpm, changing the medium each hour.

2.8.2. Budesonide quantification

Quantification was performed using a Purospher C18 endcapped analytical column with particles of 5 µm (L 150 mm, d.i. 4 mm) and a mobile phase in an isocratic mode composed of a mixture of water and methanol (35/65 % v/v) at a flow rate of 1 mL/min. The column temperature was kept constant at 30 °C, 50 µL of the samples were injected at room temperature, the chromatographic run time was set to 10 min and the detection wavelength was 245 nm. The retention time of BUD was 7.4 min. The calibration solutions consisted of 6 concentrations: 100, 20, 10, 5, 0.5 and 0.25 µg/mL. Sample preparation consisted of a 10-fold dilution in methanol.

2.8.3. Encapsulation efficiency determination

EE was determined by measuring the concentration (µg/mL) of budesonide before (total drug) and after purification by dialysis (free drug) using the equation 4 below:

Equation 4. EE (%) determination.

$$EE(\%) = \frac{(\text{total drug} - \text{free drug})}{(\text{total drug})} x100$$

2.9. Statistics

All experiment were realized in triplicate (n = 3). A One-Way ANOVA analysis was applied with a Tukey post-test to compare three or more columns between them. An unpaired T-test was applied to compare two columns between them. The difference was considered as significant if P-value was inferior to 0.05 (*), to 0.01 (**) or to 0.001 (***).

3. Results and discussions

3.1. Optimization of the one-step liposome production and sterilization process

Previous studies (Delma et al., 2023) identified the main factors influencing the CQAs of liposomes: the pressure, the temperature, and the contact time with SC CO_2. These factors were selected as process-related parameters to be studied and the ranges of values for each factor were subsequently established. In accordance with physicochemical properties required for pharmaceutical products, a size inferior to 300 nm with a PdI inferior to 0.35 were selected as CQAs to be minimized whereas a SAL of 10^{-6} was considered as a mandatory CQA.

The 20 experiments of the experimental design (Table 2) were conducted with a high-pressure cell with liposomes composed of SPC, CHOL and DSPE-PEG_{2000} (65/30/5 % mol/mol) (formulation A, Table 1) at 5 mM.

The SAL response was binarized into "pass" for values equal to or greater than 6 log reductions of *Bacillus atropheus* spores and "fail" for values less than 6 log reductions. It can be seen that all experiments at 80 °C resulted in a "pass" SAL response and all experiments at 40 or 60 °C resulted in a "fail" response (Table 4, Appendix). The SAL response was then analyzed using a generalized linear model (GLM) for binomial regression with a logit link and a Firth correction to avoid the separation problem. Estimation of the GLM parameters confirmed that only temperature had a significant effect on SAL.

The importance of a high temperature on the sterilization with SC CO_2 is well discussed in the literature (Perrut, 2012; Shieh et al., 2009; Spilimbergo et al., 2003, Zani et al., 2013). Indeed, Zani and colleagues in their study on SC CO_2 sterilization of corticosteroids reported that an increase in temperature led to a significant increase of the microbicidal effect of SC CO_2 (Zani et al., 2013). They also demonstrated that an increase in pressure above a certain value did not significantly influence the sterilizing power of SC CO_2. While a minimum temperature of 80 °C is required to achieve sterility, this high temperature increases the risk of degradation of phospholipids and active ingredients encapsulated into liposomes. Indeed, a previous study showed that liposomes prepared using a SC CO_2 process at 80 °C and 240 bar for 30 min showed an increase in phospholipid hydrolysis and oxidation products within acceptable limits and without any change in physicochemical properties (Penoy et al., 2022). Below 9%, hydrolysis products (LPC) may even be favorable and regulate membrane fluidity (Zuidam et al., 1995). To offset the risk of degradation, studies using SC CO_2 at low temperatures for sterilization have used additives such as hydrogen peroxide (H_2O_2), peracetic acid (PAA), water (H_2O) and acetic anhydride (AC_2O). These additives make it possible to achieve sterility under milder conditions (Soares et al., 2019; Bernhardt et al., 2015), but the use of toxic additives, even in small amounts, could pose safety problems. Moreover, many additives are oxidants and are prone to oxidize components of liposome formulations (Delma et al., 2021; Grit and Crommelin, 1993;

Saetern et al., 2005). Non-toxic and non-oxidizing additives could be used to reduce the working temperature and increase the compatibility of the process with a wide variety of liposome formulations containing a wide variety of APIs.

The PdI and the Z-average size were modelled by linear mixed models. Regarding the experimental results of the design of experiments (DoE), only a few experiments allowed to achieve the CQAs (Table 4, Appendix). Figs. 2 and 3 (and Figures 7 and 8, Appendix) show the influence of process parameters on Z-average size and PdI. While the contact time and the pressure alone seem to have a slight effect on the Z-average size, their interaction seems to be important with an unfavorable area at low pressure and short contact time. Temperature seems also to have an influence with small size and PdI at low or high temperature. These results confirm our previous study which have shown that for liposome production using SC CO_2, increasing temperature allowed to obtain liposomes of small size while above certain values, an increase of pressure and contact time had little effect on size and PdI (Penoy et al., 2021). However, since the goal of the present study was to produce sterile liposomes, temperature was fixed at its high level (80 °C) and was not further optimized for the PdI or size.

A hypothesis of the explanation of the failed CQAs for Z-average size and PdI during the DoE is the unsuitability of the high-pressure cell for liposomes production. Since sterility assessment requires working under aseptic conditions, we used high pressure-cells that can be handled in an aseptic environment. However, in these high-pressure cells the agitation is insufficient and does not produce liposomes with adequate physico-chemical properties, unlike the PGSS method. Nevertheless, the size and PdI of liposomes could easily be reduced with our previously developed process using SC CO_2 as a dispersing agent with the PGSS method (Penoy et al., 2021). Indeed, this previous work allowed to determine conditions to produce liposomes of a size below 200 nm and a PdI below 0.35.

The regression models of the different responses were combined in a common profiler (Fig. 4) to enable the setting of working conditions where the targeted CQAs could be reached. As one can see, high temperature is required to achieve SAL $\leq 10^{-6}$. Considering the risk degradation of lipids and APIs at high temperature, the temperature of 80 °C was considered as a maximum for the design plan and higher temperatures were not investigated.

To minimize lipid degradation as possible, the shortest possible contact time was desired. Given the interactions between pressure and temperature, and between pressure and contact time, a high pressure of 240 bar was chosen with a short contact time of 30 min. These conditions (80 °C, 240 bar, 30 min) coincide with the condition for obtaining liposomes by PGSS as described by Penoy et al (Penoy et al., 2021). This condition was then validated for the production and sterilization of liposomes in a single step and for transfer to other liposome formulations.

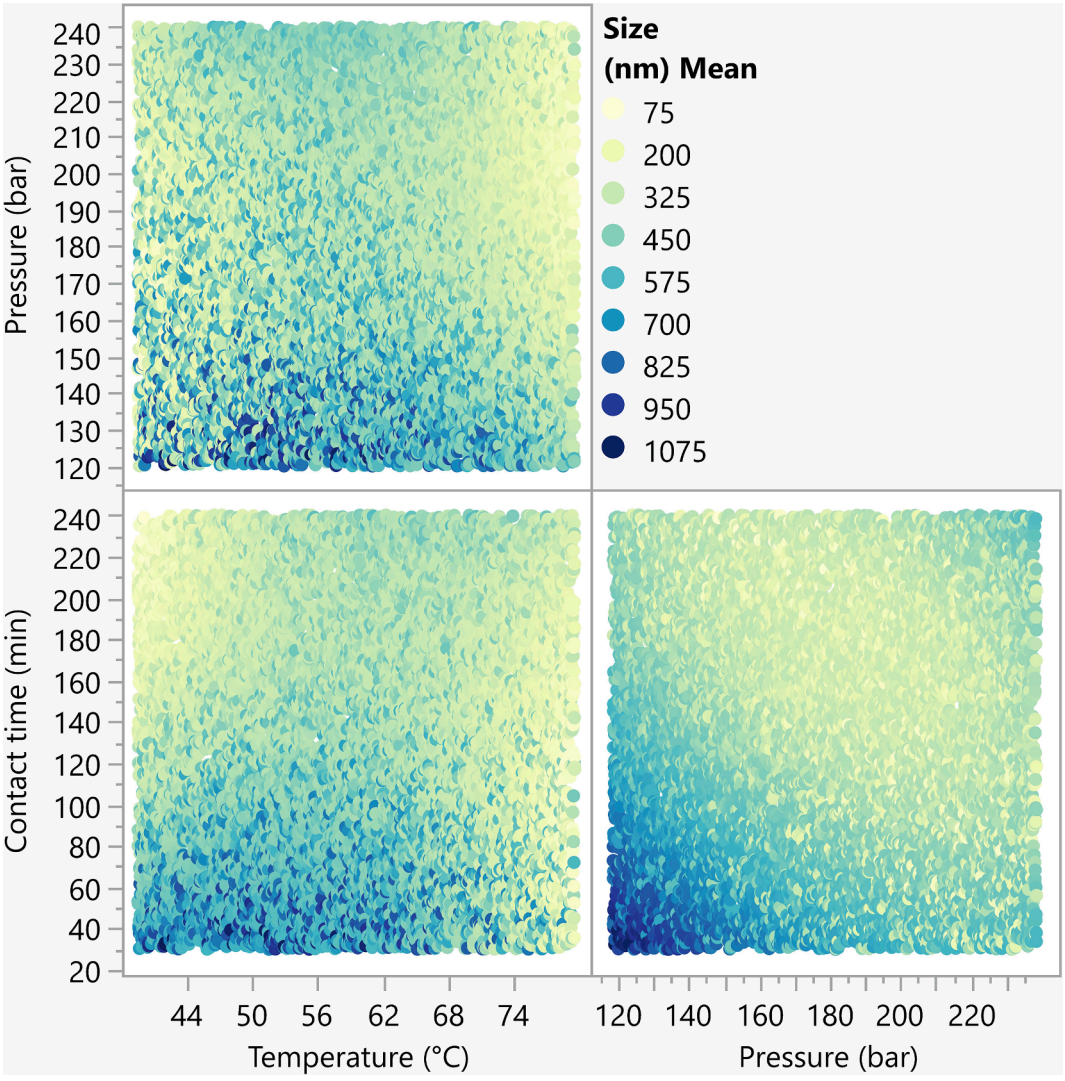

Fig. 2. Influence of temperature (°C), pressure (bar) and contact time (min) on Z-average size (nm) for liposomes (SPC/CHOL/DSPE-PEG_{2000} 65/30/5 % m/m 5mM) production. The values of the light yellow to dark blue dots are the predicted values of Z-average size (nm).

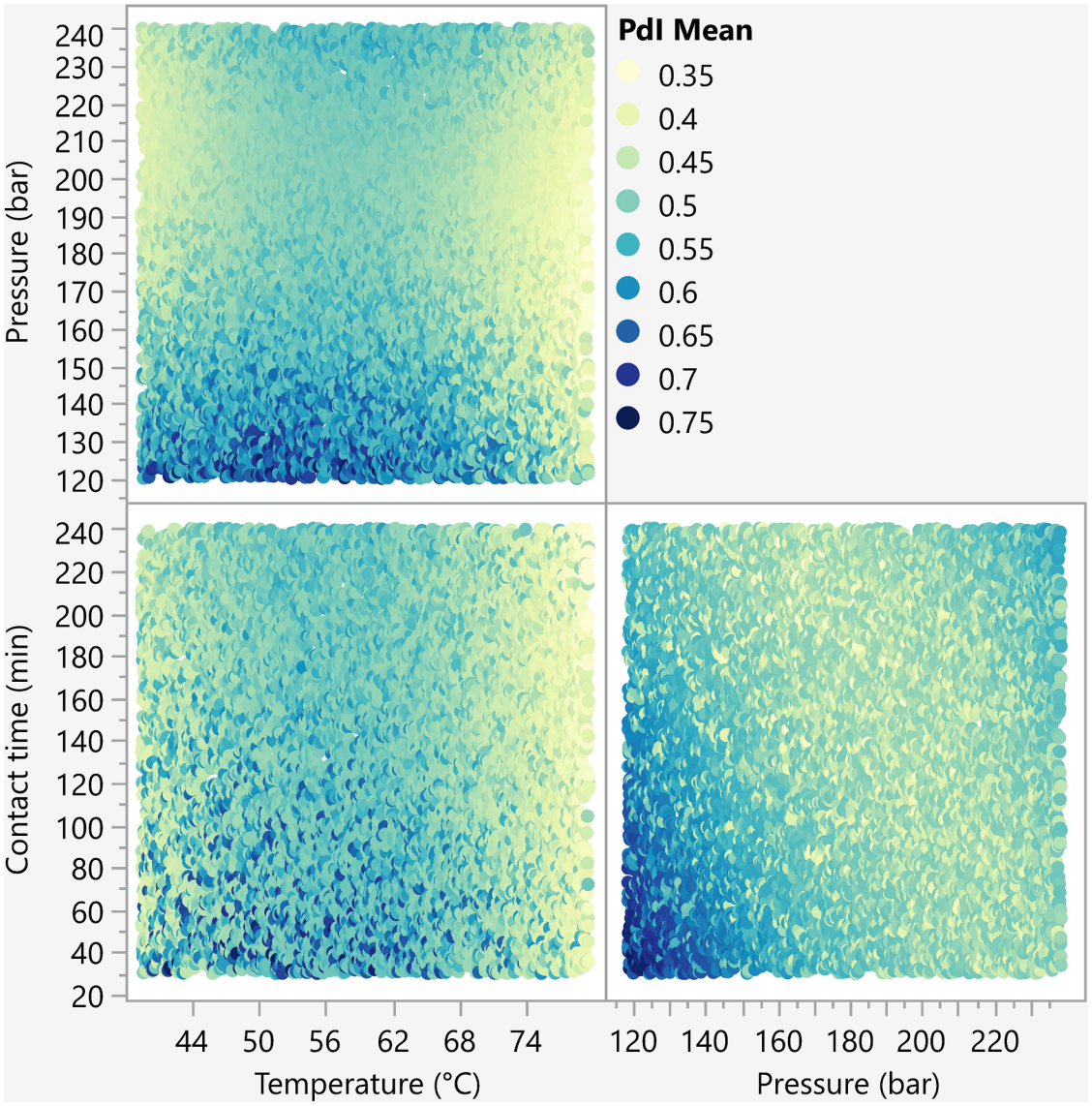

Fig. 3. Influence of the temperature (°C), pressure (bar) and contact time (min) on PdI for liposomes (SPC/CHOL/DSPE-PEG_{2000} 65/30/5 % m/m 5mM) production. The values of the light yellow to dark blue dots are the predicted values.

3.2. Validation of the process

3.2.1. Reproducibility study

To validate and assess the reproducibility of the process conditions, formulation A, composed of SPC, CHOL and DSPE-PEG_{2000} (65/30/5 %) was produced in triplicate at two lipid concentrations (at 5 and 45 mM). The results are presented in Table 3.

The results confirmed that these conditons allow to achieve the required SAL for pharmaceutical products (Log reduction > 6) for a simple PEGylated formulation (formulation A) at both 5 and 45 mM lipid concentration. These results agree with the predictions obtained with the experimental plan. Regarding the size and the PdI of liposomes, a size of less than 200 nm with a PdI around 0.30 was obtained with no significant variability. These results are consistent with our previous studies regarding the production of liposomes for drug delivery (Penoy et al., 2021). This validation confirms the development of a robust process to produce and sterilize a simple PEGylated formulation at a low and a high lipid concentration.

However, the use of a high temperature with natural and unsaturated phospholipids such as SPC, can lead to lipid degradation. For this reason, the condition was transferred to other liposome formulations composed of saturated and synthetic phospholipids and attended for different applications.

3.2.2. Transferability of the process to other liposome formulations

More complex liposome formulations were selected to evaluate the transferability and versatility of the condition. A cationic liposome formulation (Formulation B; Table 1), adapted to gene therapy and composed of DOTAP, CHOL and DOPE, was selected (Lechanteur et al., 2015). To reduce the phenomenon of phospholipid hydrolysis and oxidation, a formulation C (Table 1), composed of a synthetic phospholipids DSPC, CHOL and DSPE-PEG_{2000} was used, as well as another formulation composed of the saturated lipid, HSPC, CHOL and DSPE-PEG_{2000} (Formulation D, Table 1). Each formulation was produced with the high-pressure cell to work under aseptic conditions to determine the SAL and by the PGSS process to characterize the size and the PdI.

The validated process conditions of production and sterilization result in one-step production of liposomes with the desired Z-average size and PdI for formulation containing synthetic lipid DSPC and saturated lipid HSPC (formulation C and D, Table 1) (Fig. 5). Indeed, liposomes composed of DSPC, CHOL and DSPE-PEG_{2000} (formulation C) have a size of 164.04 ± 7.48 nm and a PdI of 0.34 ± 0.01 at 5 mM lipid

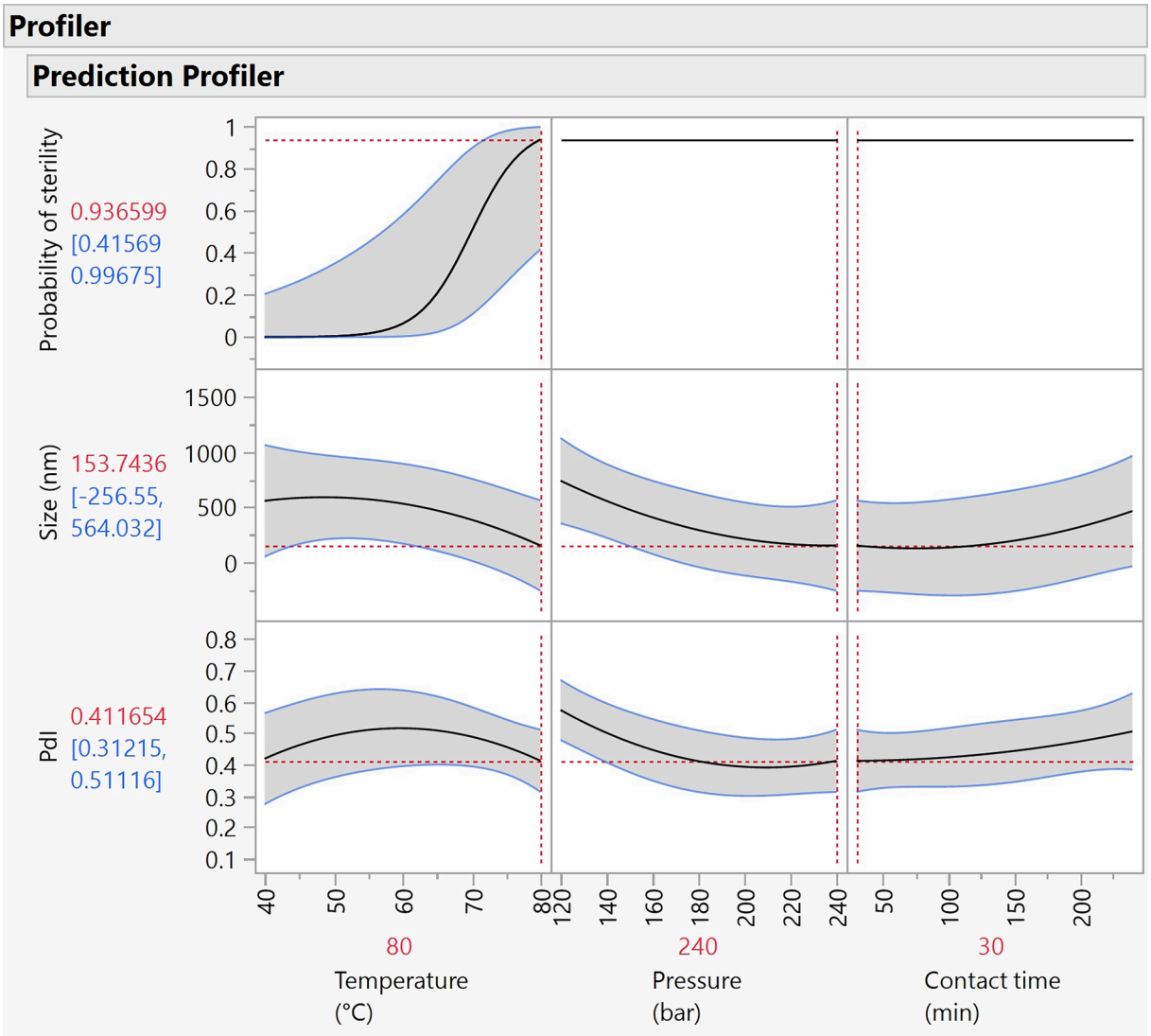

Fig. 4. DoE prediction profiler: model effects visualization of the experimental design. Red values of the responses are the predicted values for the selected parameters values together with their 95% confidence intervals in brackets.

Table 3
Size, PdI and SAL results obtained with the production and sterilization condition (80 °C; 240 bar, 30 min) with formulation A (SPC/CHOL/DSPE-PEG$_{2000}$ 65/30/5 % m/m) at 5 and 45 mM (n = 3).

Lipid concentration (mM)	Size (nm)	PdI	Log reduction
5	127.4 ± 6.9	0.31 ± 0.04	>6
45	121.7 ± 37.0	0.32 ± 0.02	>6

concentration and a size of 163.80 ± 4.92 nm with a PdI of 0.35 ± 0.03 at 45 mM lipid concentration. Liposomes composed of HSPC, CHOL and DSPE-PEG$_{2000}$ (formulation D, Table 1) have a size of 173.63 ± 2.72 nm with a PdI of 0.35 ± 0.02 at a high lipid concentration of 45 mM. Synthetic and saturated phospholipids are known to be more stable to degradation reactions such as hydrolysis and oxidation induced at high temperatures in the presence of water and carbonic acid. Indeed, in a previous study we showed that no significant degradation (hydrolysis or oxidation) was observed for the saturated phospholipids HEPC, HSPC, DPPC, DSPC and DSPE-PEG$_{2000}$ (Delma et al., 2023).

However, in the case of formulation B consisting of DOTAP, CHOL and DOPE with 45 mM lipid, higher sizes (310.3 ± 30.49 nm) and a particularly high PdI (0.44 ± 0.13) were observed. This could be explained by the high amount of cationic lipid. Penoy et al. (Penoy et al., 2021) have previously shown that the production of the DOTAP/CHOL/DOPE formulation by the PGSS process tends to produce liposomes larger than 200 nm with a PdI greater than 0.35. This could be explained by electrostatic repulsive forces that could interfere with liposome formation. However, the size of liposomes prepared at 5 mM lipid is compatible with CQAs, but the PdI remains too high for parenteral administration (201.73 ± 31.56 nm and 0.48 ± 0.09). Optimization of the conditions could be considered for this formulation.

Regarding the SAL results, each liposome formulations showed the desired SAL (log reduction > 6).

The stability of all liposome formulations prepared by PGSS and stored at 4 °C was assessed as a function of changes in the Z-average size and PdI over a period of four weeks. No significant difference was observed for any liposome formulations at the end of four weeks, demonstrating the stability of liposomes prepared with the PGSS process at the validated conditions.

3.3. *Application of the conditions to liposomes encapsulating API*

The feasibility of encapsulating a model API in liposomes using the validated condition and the impact of the encapsulated API on sterilization were investigated. Budesonide was used as a hydrophobic model

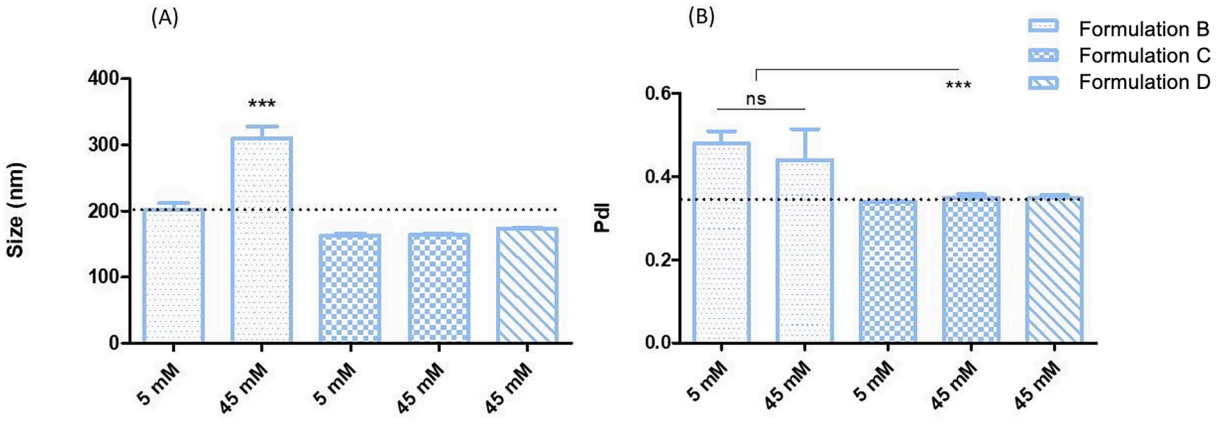

Fig. 5. (A) Z-average size (nm) and (B) PdI of liposomes formulations B, C and D produced at 5 or 45 mM lipid concentration with the SC CO_2 validated condition (80°C, 240 bars, 30 minutes).

API. This model API present a log P value of 1.91 and a poor aqueous solubility, which makes possible its encapsulation in lipid bilayers.

Encapsulation efficiency was calculated after budesonide quantification in liposomes composed of SPC or HSPC, CHOL and DSPE-PEG_{2000} (formulations A and D, Table 1) before and after purification by dialysis.

Results are presented in Fig. 6. High EE of budesonide were obtained for both formulations containing unsaturated and saturated phospholipid. The EE is nevertheless influenced by the lipid concentration. Indeed, EEs obtained at 45 mM lipids (Formulation A and D 45 mM) are significantly higher than the EE obtained at 5 mM lipids (Formulation A 5 mM). This can be explained by budesonide hydrophobicity, which is mostly entrapped into the phospholipid bilayers, thus a higher lipid concentration leads to a higher EE. This enhanced EE can be also explained by the larger population of vesicles (Eloy et al., 2014; Ullmann et al., 2021). No significance difference was observed in the EE for SPC or HSPC formulation despite the difference in degree of saturation of the lipids.

The encapsulation of budesonide has no influence on liposomes sterilization with a SAL for liposomes encapsulating budesonide $< 10^{-6}$.

Using this model API, the condition could then be transferred to other hydrophobic API, used in marketed parenteral formulations such as doxorubicin (Doxil©) (Log P value of 1.27 and poor aqueous solubility), or applied to other highly studied molecules such as cannabidiol encapsulation (Log P value of 6.3 and poor aqueous solubility) (Mihailova et al., 2022; Assadpour et al., 2023), taking into account the stability of these APIs at the temperature of 80 °C.

4. Conclusions

In this study, the possibility of using SC CO_2 for the production and sterilization of liposomes in a one-step process was evaluated. By applying a QbD strategy, this work allowed to determine the influence of the process parameters on the production and the sterilization of liposomes, and to optimize the conditions to obtain sterile liposomes with compatible physicochemical properties for drug delivery in a completely one-step process without the use of organic solvents and sterilization additives. The results demonstrated the production of liposomes with a Z-average size less than 200 nm and a PdI of less than 0.35 for most formulations with a required SAL. The SC CO_2 process conditions (80 °C, 240 bar, 30 min) allowed the production of liposomes with both low (5 mM) and high (45 mM) lipid concentration and with different lipid compositions which is very attractive from an industrial point of view. The conditions also allowed the encapsulation of a hydrophobic model drug with high EE without any influence on the sterilization process of liposomes, meaning this condition could be used for the large-scale production of concentrated batches with high EE suitable for industrial perspectives. The one-step PGSS process allows the number of production steps to be reduced compared to other conventional production methods or the industrial method currently used. This one-step liposome production and sterilization process should now be validated on a larger scale on PGSS pilot equipment located in a clean room enabling aseptic production.

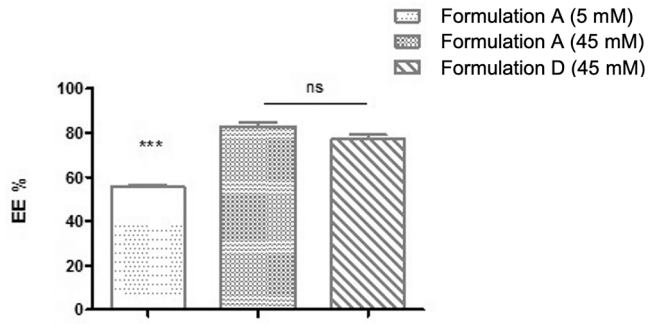

Fig. 6. EE (%) of Budesonide in liposomes formulation A at 5 and 45 mM and formulation D at 45 mM.

CRediT authorship contribution statement

Noémie Penoy: Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. **Kouka Luc Delma:** Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. **Nirmayi Homkar:** Formal analysis, Investigation, Methodology, Visualization. **Abdoul Karim Sakira:** Methodology, Writing – review & editing. **Sabrina Egrek:** Methodology, Writing – review & editing. **Rosalie Sacheli:** Methodology, Writing – review & editing. **Pierre-Yves Sacré:** Methodology, Software, Writing – review & editing. **Bruno Grignard:** Methodology, Writing – review & editing. **Marie-Pierre Hayette:** Methodology, Writing – review & editing. **Touridomon Issa Somé:** Methodology, Writing – review & editing. **Rasmané Semdé:** Conceptualization, Funding acquisition, Supervision. **Brigitte Evrard:** Conceptualization, Funding acquisition, Supervision. **Géraldine Piel:** Conceptualization, Methodology, Supervision, Writing – review & editing.

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.

Acknowledgements

The authors would like to acknowledge the Professors Michel Frederich and Joëlle Leclercq for their precious material and technical support, the laboratory of Microbiology for the material and technical support and the FEDER funds (PHARE project) as well as the Academy of Research and Higher Education – Committee for Development Cooperation (ARES-CCD) for their financial assistance.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpharm.2024.123769.

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