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(54) Title: CONTINUOUS PRODUCTION OF LIPOSOMES WITH SUPERCRITICAL CARBON DIOXIDE

(57) Abstract: A method of producing liposomes includes steps of: (a) continuously pumping an aqueous phase comprising phospholipids and at least one target compound and carbon dioxide into an in-line mixer, to produce a mixture; (b) transferring the mixture to a pressure vessel maintained at supercritical conditions; and (c) continuously drawing off and depressurizing an output mixture stream from the pressure vessel, producing liposomes.

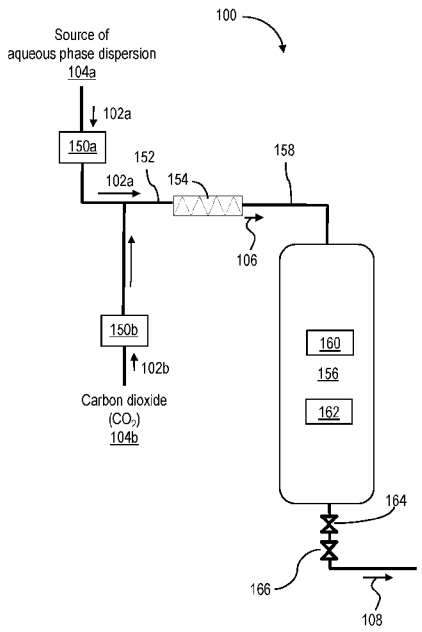

FIG. 1

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CONTINUOUS PRODUCTION OF LIPOSOMES WITH SUPERCRITICAL CARBON DIOXIDE

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] The present application claims the priority benefit of United States Provisional Application 63/395,461 filed on August 5, 2022, the entire contents of which are incorporated herein by reference.

FIELD OF THE INVENTION

[0002] This invention relates to a method based on the use of supercritical carbon dioxide (SC-CO_2) for the continuous production of liposomes.

BACKGROUND OF THE INVENTION

[0003] Liposomes are colloidal spherical vesicles formed by self-assembling of phospholipids in contact with water and composed of an aqueous core enclosed by one or more lipid bilayers. Liposomes have excellent properties for their use as carriers and delivery systems of drugs and many other compounds for application in pharmaceutical, cosmetic, food and natural health product industries. They are biocompatible, biodegradable, non-toxic, non-immunogenic and they can entrap both lipophilic and hydrophilic bioactives and drugs.

[0004] More generally, liposome formation is an ever-expanding field that requires environmentally friendly, economical and versatile production techniques for the incorporation of compounds with varied chemical structures and water solubilities, and the use of a wide variety of phospholipids.

[0005] Natural, synthetic and semi-synthetic phospholipids, such as phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and phosphatidylglycerol, have been extensively used for liposome formation. More recently, the application of surface-modified liposomes using glycolipids, polyalkylene oxide derivatives, PEGylated forms and other hydrophilic polymers, has at least partly overcome or reduced some of the drawbacks

associated with phospholipids from natural sources, as for example, their relative instability and their rapid clearance from the blood circulatory system.

[0006] To date, a number of methods have been proposed for the production of liposomes, targeting the formation of stable, small (generally in the nanometer scale) and homogeneous particles containing a high load of the targeted compound(s). Conventional methods for liposome formation, such as thin-film hydration, reverse-phase evaporation, solvent injection, proliposome-liposome and detergent removal methods, require the use of organic solvents and additional processing steps to remove the solvent, which increases the operating costs and the environmental impact, and generates residues in the final product. In addition, these processes cannot operate in continuous mode.

[0007] In general, these methods are characterized by the difficulty to control the particle size, leading to the formation of big particles, heterogeneous size distributions and low reproducibility of the results, and therefore different downsizing techniques, such as extrusion, filtration, sonication and pressure homogenization, are commonly applied to the liposomal phase to reduce the size and the polydispersity of particles, further increasing the operating costs and the environmental impact.

[0008] Microfluidic methods, such as pulsed jetting, double emulsion templating, droplet emulsion transfer and microfluidic hydrodynamic focusing, have been proposed as alternatives to conventional methods. In general, these methods provide a better control of the particle size and enable the formation of monodisperse particles. However, the use of organic solvents is still necessary and this technology has not been implemented at industrial scale for the mass production of liposomes.

[0009] Supercritical carbon dioxide (SC-CO_2) methods have also been proposed as an alternative to overcome the drawbacks associated with the use of conventional techniques. SC-CO_2 is a nonflammable, non-toxic, non-corrosive and inexpensive solvent. Its low critical temperature (31.1 °C) prevents the degradation of bioactive compounds and its solvent power and fast diffusion rates at supercritical conditions can be exploited to create highly

supersaturated systems, which quickly evolve towards precipitation of small particles and homogeneous products.

[0010] A number of methods using SC-CO_2 have been proposed for liposome production. However, organic solvents are commonly used in these methods in order to improve the contact between the substances and obtain particles with low particle sizes and homogeneous size distributions.

[0011] Further, the majority of SC-CO_2 methods operate in batch or semi-continuous mode, so the continuous production of liposomes cannot be accomplished. Therefore, even though SC-CO_2 technology offers many advantages with respect to other liposome formation techniques, existing methods still present important limitations for an efficient large-scale liposome production.

[0012] There remains a need in the art for a continuous method of producing liposomes with SC-CO_2, which may mitigate some or all of the difficulties in the prior art.

SUMMARY OF THE INVENTION

[0013] In one aspect, this disclosure relates to a process operating in continuous mode, where the raw materials (CO_2, phospholipids and at least one target compound dispersed or dissolved in water) are continuously introduced into a pressure vessel maintained at supercritical conditions, and liposomes are formed and collected throughout the processing period. In preferred embodiments, this process does not require the use of organic solvents and a solvent-free aqueous product may be obtained by simple depressurization of SC-CO_2. In contrast to conventional methods, embodiments of this invention may allow the formation of liposomes with sizes smaller than 200 nm, narrow particle size distributions and/or high encapsulation efficiencies in a single step.

[0014] In some embodiments, the process may be implemented with relatively low pressure (for example 100 bar) and temperature (for example 40 °C). Optionally, a stirring mechanism may be used in the pressure vessel to improve the mixing between the components. Optionally,

a nozzle at the system outlet may be used to reduce the size and size distribution of the particles. The products resulting from operation of a process described herein are liposomes with sizes in the range of nanometer scale and narrow particle size distributions.

[0015] In some embodiments, the process is not limited by the type of target compound, which may comprise any lipophilic, hydrophilic or intermediate polarity range drug and/or bioactive compound.

[0016] In some embodiments, the process begins with the pumping of the aqueous phase containing the phospholipid(s) and at least one target compound and CO_2 stream into a pressure vessel, while the system outlet is closed. As aqueous phase and CO_2 streams are pumped into the pressure vessel, the pressure vessel contents are pressurized and heated up to the supercritical operating conditions. Preferably, the aqueous phase and the CO_2 are thoroughly mixed before entering the pressure vessel. Once the set pressure and temperature are established, the mixture is allowed to leave the system and depressurize. Depressurization, such as through a micrometering valve, will cause the rapid release of CO_2 as gas, leading to self-assembly of phospholipids to form liposomes in the aqueous phase. The collection rate of the aqueous phase containing the dispersed liposomes is set based on the flow rates of feed aqueous phase and CO_2 streams to keep the pressure inside the system substantially constant throughout the process.

[0017] Embodiments of the present invention may be implemented by modifying existing commercial processes and other methods proposed as alternatives to them. The versatility of the present invention offers the possibility of entrapping both hydrophilic and lipophilic compounds, and using a variety of phospholipids as carriers for the continuous production of liposomes.

BRIEF DESCRIPTION OF THE DRAWINGS

[0018] In the drawings, like elements may be assigned like reference numerals. The drawings are not necessarily to scale, with the emphasis instead placed upon the principles of

the present invention. Additionally, each of the embodiments depicted are but one of a number of possible arrangements utilizing the fundamental concepts of the present invention.

[0019] Figure 1 is an example system for continuously producing liposomes with supercritical carbon dioxide (SC-CO_2).

[0020] Figure 2 is a process flow for an example method for continuously producing liposomes with supercritical carbon dioxide (SC-CO_2).

[0021] Figure 3A shows a plot of the size (P_S) and size distribution (expressed in terms of polydispersity index, PdI) of empty liposomes in an output mixture, obtained at different filling cycles using a pressure vessel operating at conditions of 100 bar and 40 °C, and a pump used for pumping an aqueous phospholipid phase into the vessel at a 3.0 g/min flow rate.

[0022] Figure 3B shows the size (P_S) and size distribution (expressed in terms of polydispersity index, PdI) of empty liposomes in an output mixture, obtained at different filling cycles using a pressure vessel operating at conditions of 100 bar and 40 °C, and a pump used for pumping an aqueous phospholipid phase into the vessel at a 7.0 g/min flow rate.

[0023] FIG. 4A shows a plot of the encapsulation efficiency (EE) of coQ10-loaded liposomes obtained using the disclosed invention versus a conventional thin-film hydration (TFH) method, over the 35 days of storage at 4 °C.

[0024] FIG. 4B shows a plot of the particle size (Ps) of coQ10-loaded liposomes obtained using the disclosed invention versus the conventional TFH method, over 35 days of storage at 4 °C.

[0025] FIG. 4C shows a plot of the polydispersity index (PdI) of coQ10-loaded liposomes obtained using the disclosed invention versus the conventional TFH method, over 35 days of storage at 4 °C.

DETAILED DESCRIPTION OF EMBODIMENTS OF THE INVENTION

[0026] This disclosure relates generally to a method based on the use of supercritical carbon dioxide (SC-CO_2) for the continuous production of liposomes, preferably without the addition of organic solvents.

[0027] I. DEFINITIONS.

[0028] Any term or expression not expressly defined herein shall have its commonly accepted definition understood by a person skilled in the art.

[0029] "Phospholipid" or "phospholipids" are polar lipids that consist of two fatty acids, a glycerol unit and a phosphate group which is esterified to an organic molecule, and includes all natural, synthetic and semi-synthetic phospholipids, such as phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and phosphatidylglycerol, glycolipids, polyalkylene oxide derivatives, PEGylated forms and forms with other hydrophilic polymers.

[0030] "Target compound" means any molecule which is intended to be trapped in the liposomes, and may include hydrophilic, lipophilic and/or intermediate polarity range molecules.

[0031] II. OVERVIEW.

[0032] In one aspect, this disclosure relates to a novel process using supercritical carbon dioxide (SC-CO_2) to continuously produce liposomes, which may be suitable for the encapsulation of both hydrophilic and hydrophobic compounds. The methods described herein may be scalable and relatively easy to implement. The process may be configured as a stand-alone process, or may be integrated as a new unit operation into large multipurpose plants, employing other SC-CO_2 unit operations such as extraction.

[0033] Surprisingly, the inventors have discovered that stable liposomes with particle sizes below 300 nm, narrow particle size distributions (polydispersity index less than 0.3) and encapsulation efficiencies higher than 90% can be obtained at relatively mild pressures and

temperatures and under near-equilibrium conditions. Near-equilibrium conditions are preferred because it allows a substantial reduction of: *i)* the amount of CO_2 required for the formation of liposomes, and *ii)* the processing time, which, in turn, can be exploited for the design of simple processes.

[0034] SC-CO_2 is mainly used as a propellant as well as a solute since it dissolves into the aqueous and lipid phases, so it is not required in large quantities. In addition, preferred embodiments do not require the use of organic solvents and thus a solvent-free product is obtained. Thus, in some embodiments, the use of an organic solvent is specifically excluded. If hydrophobic substances are the target molecule, then they may be introduced as a phase in the water phase, which enhances the technical simplicity of this process.

[0035] III. SYSTEM.

[0036] Figure 1 shows an example system (100) for continuous production of liposomes with supercritical carbon dioxide (SC-CO_2).

[0037] As shown, system (100) includes pumps (150a), (150b) which are used to provide continuous pumping of: (i) an aqueous phase (102a) containing combined phospholipid(s) and at least one target compound, from a source (104a), and (ii) CO_2 (102b), from a source (104b). The aqueous phase (102a) and the CO_2 (102b) is pumped through a first line (152) into an in-line mixer (154), where the aqueous phase (102a) and CO_2 (102b) are mixed to form a mixture (106). The mixer (154) may comprise any mixer commonly used for such a purpose. Mixture (106) enters into a pressure vessel (156) via conduit line (158).

[0038] In preferred embodiments, the whole system after the pumps is under pressure including the in-line mixer. An on/off valve (164) at the exit is initially closed. On startup, the heaters (not shown) are used to bring the temperature up to the desired level. The heaters may be applied to each of the in-line mixer (154), the conduit (158) and the pressure vessel (156). The pumps (150a, 150b) are then turned on and the pressure in the system increases until a target pressure is reached. When temperature and pressure are stable at the target level, the

on/off valve (164) is opened and, optionally, a micrometering valve or nozzle (166) is used to adjust the exit flow rate to maintain the desired pressure within the pressure vessel.

[0039] The bulk mixture coming out from the pressure vessel is thus depressurized and is collected, whereby the output mixture (108) includes liposomes formed during depressurization.

[0040] Preferably, a target compound, in the aqueous phase (102a), is solubilized or dispersed in water without requiring any pre-treatment, other than stirring together with the phospholipid(s). Nevertheless, depending on their chemical nature and the characteristics of the pump system used, the application of mild heat or filtration prior to pumping might be necessary or desirable. In some examples, the pressure vessel (156) can incorporate a stirring system (160), such as paddle and propeller agitators, or be packed with any material (162), which could improve the mixing and contact between the substances. For example, the packing material (162) may comprise glass beads or the like.

[0041] In preferred embodiments, the systems and methods described herein do not require the use of a nozzle or a related system at the outlet, which improves the process simplicity. Nevertheless, a nozzle (166) or the like may optionally be incorporated, as a nozzle could promote a size reduction of the liposomes obtained upon depressurization.

[0042] IV. METHOD.

[0043] Figure 2 shows a process flow for an example method (200) for continuously producing liposomes with supercritical carbon dioxide (SC-CO).

[0044] As shown, at step (202), the method involves continuously pumping (i) an aqueous phase (102a) comprising a phospholipid and at least one target compound, and (ii) CO_2 (102b) through an in-line mixer (154) and then into a pressure vessel. The mixture may be heated in the in-line mixer, in the transfer conduit (158) and in the pressure vessel (156).

[0045] As noted previously, preferably, the target compound(s) are solubilized or dispersed in water without requiring any pre-treatment other than stirring together with the

phospholipid(s). Nevertheless, depending on their chemical nature and the characteristics of the pump or propulsion system used, the application of heat or filtration prior to pumping might be necessary or preferred. For example, a heater or a filter can be positioned prior to the pump (150a) (Figure 1) to apply the heat or filtration, respectively.

[0046] At step (204), the mixture (158) is introduced to a pressure vessel (156), which is operated at supercritical conditions. In some embodiments, the process temperature may preferably be below 60 °C and more preferably be about 40° C. The process pressure may preferably be below 300 bar, and more preferably be in the range of 100 to 200 bar. Thus, in some embodiments, a process for producing liposomes comprises a single step process operating in continuous mode, where the raw materials are continuously introduced into the system and liposomes are simultaneously formed and collected during the processing period.

[0047] Step (206) involves continuously drawing-off a stream of the mixture from the pressure vessel (156), through valve (164) thereby producing a depressurized output mixture (108) with liposomes formed therein. In some examples, a micrometering valve or a nozzle (166) is used to promote a size reduction of the particles obtained upon depressurization.

[0048] In some embodiments, the process begins, at step (202), with the pumping of the aqueous phase (102a) and CO_2 (102b) streams at specific flow rates, while the on/off valve (164) is closed. The flow rates and/or pressure can be controlled via controlling pumps (150a, 150b).

[0049] In preferred embodiments, a sufficient amount of C02 is used to saturate the mixture in the pressure vessel.

[0050] As the aqueous phase (102a) and CO_2 (102b) streams are pumped through the mixer and into the pressure vessel, the vessel (156) is pressurized and heated up to the operating conditions. Once the set pressure and temperature are established, at step (206), the on/off valve (164) is opened and the mixture (108) is allowed to leave the system and depressurized through the micrometering valve/nozzle (166) while the input streams (102a), (102b) are continuously pumped in.

[0051] The depressurization causes the rapid release of CO_2 as gas and the liposomes are formed at a specific rate. The collection rate of the aqueous mixture (108) at the exit, containing the dispersed liposomes will be set based on the flow rate of aqueous phase and CO_2 streams at the inlet to keep the pressure in the system (100) constant during the process.

[0052] In some embodiment, this invention allows the size of the liposomes to be tuned by modifications in the processing conditions. For example, a decrease in the water flow rate and an increase in pressure leads to a smaller particle size. Depending on the type of phospholipid used and its phase transition temperature, an increase in temperature would also lead to a decrease in particle size.

[0053] V. EXAMPLES.

[0054] The following examples illustrate specific elements of exemplary embodiments, which are not intended to be limiting of the claimed invention.

[0055] Liposomes, prepared using soy lecithin as the phospholipid source, were obtained using a pressure vessel (156) being operated at operating conditions comprising: (i) pressures from 100 to 200 bar, and (ii) temperatures of 40 and 60 ºC, and further obtained by operating the pump (150a) to allow for aqueous phase flow rates of 3.0, 5.0 and 7.0 g/min. The aqueous phase flow rates could be adjusted and controlled by adjusting the operation of pump (150a).

[0056] During conducted experiments, the mass of water phase, pumped in and out of the system, was recorded for each experimental condition to determine the process mass balance. It was determined that the process mass balance was constant (less than 6% error) and thereby demonstrated that steady state was reached, a requirement necessary for a continuous production.

[0057] Plots (300a), and (300b) in Figures 3A and 3B, respectively, shows the size (Ps) (302) and size distribution (304) (expressed in terms of polydispersity index, PdI) of empty liposomes (no compound entrapped) obtained, in output mixture (108) (Figure 1), over time at

operating conditions for vessel (156) of 100 bar and 40 °C, and two different water flow rates for the pump (150a) (3.0 and 7.0 g/min), respectively.

[0058] In particular, plot (300a) (Figure 3A) shows the size (Ps) (302) and size distribution (PdI) (304) of empty liposomes obtained, for different filling cycles, at vessel operating conditions of 100 bar and 40 °C, and a pump (150a) operating to allow a flow rate of 3.0 g/min for the aqueous phase.

[0059] Plot (300b) (Figure 3B) shows the size (Ps) (302) and size distribution (PdI) (304) of empty liposomes obtained, at different filling cycles, at vessel operating conditions of 100 bar and 40 °C, and a pump (150a) operating to allow a flow rate of at 7.0 g/min for the aqueous phase.

[0060] As shown, the Ps (302) and PdI (304) of the liposomes were stable at both conditions after more than 1.6 filling cycles.

[0061] In this case, the term "filling cycle" (filling cycles = volume of aqueous phase pumped / total volume of the system) refers to the number of times that the system was filled up with fresh aqueous phospholipid phase during the production of liposomes. Filling cycle of 1 indicates that a volume of fresh aqueous phospholipid phase equivalent to the system volume was pumped in, replacing the previous volume of water contained in the system.

[0062] Table 1 show the size (Ps) (nm) and size distribution (expressed in terms of polydispersity index, PdI) of liposomes obtained using soy lecithin, where the concentration of lecithin in water (i.e., aqueous phase (102a)) was 20 mM (13.56 mg/mL). In particular, Table 1 shows Ps and PdI of empty liposomes obtained after 1.6 filling cycles at different experimental conditions.

[0063] As shown, Ps as low as 120 nm were obtained (200 bar, 40 °C, 3 g/min). A decrease in the aqueous phase flow rate and an increase in pressure led to a smaller particle size. PdI is an important parameter in particle formation and polymer science and values considerably

lower than 0.3 were obtained indicating a narrow particle size distribution and, therefore, homogenous vesicles in terms of particle size.

[0064] To that end, achieving stable Ps and PdI values at increasing filling cycles is not trivial since continuous processing requires that conditions remain constant as the processing time progresses.

Experimental condition	Ps (nm)	PdI
100 bar, 40 °C, 3 g/min	135	0.229
200 bar, 40 °C, 3 g/min	120	0.218
100 bar, 40 °C, 7 g/min	154	0.229
100 bar, 40 °C, 3 g/min, coQ10	197	0.281
200 bar, 40 °C, 3 g/min, anthocyanin	168	0.242

Table 1 - Particle size (Ps) and size distribution (PdI) of empty liposomes obtained after 1.6 filling cycles, and liposomes containing the lipophilic compound coenzyme Q10 (coQ10) and the hydrophilic compound anthocyanin, obtained at different pressures (bar) and aqueous phase flow rates (g/min) for the aqueous phase pump.

[0065] The encapsulation of two bioactives in liposomes, the lipophilic compound coenzyme Q10 (coQ10) and the hydrophilic compound anthocyanin, both having antioxidant activity, was also carried out (data presented in Table 1).

[0066] The amount of coQ10 assayed was 1.7 mM (1.47 mg/mL of water phase) and the process was performed at low pressure and temperature (100 bar, 40 °C, 3 g/min of water flow rate).

[0067] The encapsulation efficiency (EE = mass of coQ10 encapsulated / mass of coQ10 assayed x 100) obtained was 97.5%; practically the entire amount of coQ10 assayed was entrapped in the liposomes.

[0068] The bioactive loading (mass of coQ10 encapsulated / mass of phospholipids used x 100) was 11.4%; therefore, this process allowed a high loading of the compound in the liposomes obtained.

[0069] The Ps and PdI values of the coQ10-loaded liposomes obtained were, respectively, 197 nm and 0.281.

[0070] In the case of anthocyanin, the amount assayed was 2.5 mM (1.17 mg/mL) and the Ps and PdI obtained were, respectively, 168 nm and 0.242. Liposomes with a low Ps (below 200 nm) and PdI (below 0.3) are especially desirable for drug delivery applications and both values can be obtained by the present invention in a single step, without any further processing.

[0071] In addition, the stability under storage at 4 °C of coQ10-loaded liposomes was evaluated (see e.g., Figures 4A, 4B and 4C). This evaluation involved comparing, (i) coQ10-loaded liposomes obtained using the disclosed method (e.g., Figure 2), versus (ii) coQ10-loaded liposomes obtained using conventional thin-film hydration (TFH) method. The TFH method is a common batch process used for liposome formation that requires the use of organic solvents.

[0072] FIG. 4A shows a plot (400a) of the encapsulation efficiency (EE) of coQ10-loaded liposomes obtained using the disclosed invention (402a) versus the conventional TFH method (404a), over the 35 days of storage at 4 °C. Encapsulation efficiency (EE) is equal to mass of coQ10 encapsulated / mass of coQ10 assayed x 100.

[0073] FIG. 4B shows a plot (400b) of the particle size (Ps) of coQ10-loaded liposomes obtained using the disclosed invention (402b) versus the conventional TFH method (404b), over 35 days of storage at 4 °C.

[0074] FIG. 4C shows a plot (400c) of the polydispersity index (PdI) of coQ10-loaded liposomes obtained using the disclosed invention (402c) versus the conventional TFH method (404c), over 35 days of storage at 4 °C.

[0075] As shown in plots (400a) – (400c), the EE, Ps and PdI of liposomes obtained using the disclosed invention remained stable after 35 days of storage, as opposed to the values from liposomes obtained using the conventional TFH method. The obtaining of constant EE, Ps and PdI values is an indicator of liposome stability, given that liposomes are thermodynamically

unstable particles and some phenomena occurring over time, such as the leakage of the entrapped compounds and the coalescence of particles, will finally lead to a variation in all these values. Moreover, the Ps and PdI values of liposomes obtained by this invention were lower than those obtained by TFH.

[0076] VI. EXEMPLARY ASPECTS.

[0077] In view of the described apparatuses, and methods and variations thereof, certain more particularly described aspects of the invention are presented below. These particularly recited aspects should not however be interpreted to have any limiting effect on any different claims containing different or more general teachings described herein, or that the "particular" aspects are somehow limited in some way other than the inherent meanings of the language literally used therein.

[0078] Aspect 1A. A method of continuously producing liposomes, comprising the steps of:

(a) continuously pumping an aqueous phase comprising a phospholipid and at least one target compound, and CO_2, and mixing to produce a mixture;

(b) transferring the mixture to a pressure vessel and maintaining the mixture at temperature and pressure conditions above the critical point of CO_2; and

(c) continuously drawing off and depressurizing a output mixture stream from the pressure vessel, producing liposomes.

[0079] Aspect 1B. A system for continuously producing liposomes, comprising:

(a) a first pump for continuously pumping an aqueous phase comprising a phospholipid and at least one target compound;

(b) a second pump for continuously pumping carbon dioxide (CO_2);

(c) an in-line mixer receiving the aqueous phase and the CO_2, and producing a mixture; and

(d) a pressure vessel for receiving the mixture, and operable at temperature and pressure conditions above the critical point of CO_2, the pressure vessel having an output valve for continuously releasing a stream of the mixture.

[0080] Aspect 2. The method of Aspect 1A and/or the system of Aspect 1B, wherein organic solvent is not employed to produce liposomes.

[0081] Aspect 3. The method of Aspect 1A, the system of Aspect 1B, and/or Aspect 2, wherein the pressure vessel is operated at conditions comprising: (a) a temperature below about 60° C, preferably about 40° C; and/or (b) a pressure below about 300 bar, preferably below about 200 bar.

[0082] Aspect 4. The method of Aspect 1A, the system of Aspect 1B, and/or any one of Aspects 2 to 3, wherein the at least one target compound is solubilized or dispersed in water without any pre-treatment other than stirring together with the phospholipid.

[0083] Aspect 5. The method of Aspect 1A, the system of Aspect 1B, and/or any one of Aspects 2 to 4, wherein the pressure vessel comprises an optional stirring system, such as a paddle or propeller agitator, or is packed with any material to improve the mixing and contact between the substances.

[0084] Aspect 6. The method of Aspect 1A, the system of Aspect 1B, and/or any one of Aspects 2 to 5, wherein the pressure vessel comprises a micrometering valve or an outlet nozzle to promote a size reduction of the particles obtained upon depressurization.

[0085] Aspect 7. The method of Aspect 1A, the system of Aspect 1B, and/or any one of Aspects 2 to 6, wherein the resulting liposomes have: (a) a size (Ps) less than about 200 nm; and/or (b) a size distribution (PdI) of less than about 0.30.

[0086] Aspect 8. The method of Aspect 1A, the system of Aspect 1B, and/or any one of Aspects 2 to 7, wherein the at least one target compound comprises one or more of a lipophilic, hydrophilic or intermediate polarity range drug and/or a bioactive compound.

[0087] Aspect 9. The method of Aspect 1A, the system of Aspect 1B, and/or any one of Aspects 2 to 8, wherein the aqueous phase is pumped at flow rates of 3.0, 5.0 or 7.0 g/min.

[0088] Aspect 10. The method of Aspect 1A, the system of Aspect 1B, and/or any one of Aspects 2 to 9, wherein heat and/or filtration is applied to the aqueous phase, using a heater and/or filter, respectively, positioned prior to the pumping of the aqueous phase.

[0089] Aspect 11. A method of continuously producing liposomes, comprising or consisting essentially of any combination of steps, elements or features disclosed herein.

[0090] Aspect 12. A system for of continuously producing liposomes, comprising any combination of steps, elements or features disclosed herein.

[0091] VII. INTERPRETATION.

[0092] The corresponding structures, materials, acts, and equivalents of all means or steps plus function elements in the claims appended to this specification are intended to include any structure, material, or act for performing the function in combination with other claimed elements as specifically claimed.

[0093] References in the specification to "one embodiment", "an embodiment", etc., indicate that the embodiment described may include a particular aspect, feature, structure, or characteristic, but not every embodiment necessarily includes that aspect, feature, structure, or characteristic. Moreover, such phrases may, but do not necessarily, refer to the same embodiment referred to in other portions of the specification. Further, when a particular aspect, feature, structure, or characteristic is described in connection with an embodiment, it is within the knowledge of one skilled in the art to affect or connect such module, aspect, feature, structure, or characteristic with other embodiments, whether or not explicitly described. In other words, any module, element or feature may be combined with any other element or

feature in different embodiments, unless there is an obvious or inherent incompatibility, or it is specifically excluded.

[0094] It is further noted that the claims may be drafted to exclude any optional element. As such, this statement is intended to serve as antecedent basis for the use of exclusive terminology, such as "solely," "only," and the like, in connection with the recitation of claim elements or use of a "negative" limitation. The terms "preferably," "preferred," "prefer," "optionally," "may," and similar terms are used to indicate that an item, condition or step being referred to is an optional (not required) feature of the invention.

[0095] The singular forms "a," "an," and "the" include the plural reference unless the context clearly dictates otherwise. The term "and/or" means any one of the items, any combination of the items, or all of the items with which this term is associated. The phrase "one or more" is readily understood by one of skill in the art, particularly when read in context of its usage.

[0096] The term "about" can refer to a variation of ± 5%, ± 10%, ± 20%, or ± 25% of the value specified. For example, "about 50" percent can in some embodiments carry a variation from 45 to 55 percent. For integer ranges, the term "about" can include one or two integers greater than and/or less than a recited integer at each end of the range. Unless indicated otherwise herein, the term "about" is intended to include values and ranges proximate to the recited range that are equivalent in terms of the functionality of the composition, or the embodiment.

[0097] As will be understood by one skilled in the art, for any and all purposes, particularly in terms of providing a written description, all ranges recited herein also encompass any and all possible sub-ranges and combinations of sub-ranges thereof, as well as the individual values making up the range, particularly integer values. A recited range includes each specific value, integer, decimal, or identity within the range. Any listed range can be easily recognized as sufficiently describing and enabling the same range being broken down into at least equal halves, thirds, quarters, fifths, or tenths. As a non-limiting example, each range discussed herein can be readily broken down into a lower third, middle third and upper third, etc.

[0098] As will also be understood by one skilled in the art, all language such as "up to", "at least", "greater than", "less than", "more than", "or more", and the like, include the number recited and such terms refer to ranges that can be subsequently broken down into sub-ranges as discussed above. In the same manner, all ratios recited herein also include all sub-ratios falling within the broader ratio.

CLAIMS

1. A method of continuously producing liposomes, comprising the steps of:

(a) continuously pumping an aqueous phase comprising a phospholipid and at least one target compound, and CO_2 into a pressure vessel;

(b) creating and maintaining temperature and pressure conditions above the critical point of CO_2 within the pressure vessel; and

(c) continuously drawing off and depressurizing a output mixture stream from the pressure vessel, producing liposomes.

2. The method of claim 1 wherein the aqueous phase and the CO_2 are mixed together prior to entering the pressure vessel.

3. The method of claim 1 or 2, wherein the method does not employ an organic solvent to produce liposomes.

4. The method of claim 1, 2 or 3, wherein

(a) the temperature condition is a temperature below about 60° C, preferably about 40° C; and/or

(b) the pressure condition is a pressure below about 300 bar, preferably below about 200 bar.

5. The method of any one of claims 1 to 4, wherein the at least one target compound is solubilized or dispersed in water without any pre-treatment other than stirring together with the phospholipid.

6. The method of any one of claims 1 to 5, wherein the pressure vessel comprises a stirring system, such as a paddle or propeller agitator, and/or is packed with any material to improve the mixing and contact between the phospholipid and the at least one target compound and supercritical CO_2.

7. The method of any one of claims 1 to 6 wherein the pressure vessel comprises an outlet valve and/or a nozzle to promote a size reduction of the particles obtained upon depressurization.

8. The method of any one of claims 1 to 7, wherein the resulting liposomes have:

(a) a size (Ps) less than about 200 nm; and/or

(b) a size distribution (PdI) of less than about 0.30.

9. The method of any one of claims 1 to 8, wherein heat and/or filtration is applied to the aqueous phase, prior to pumping the aqueous phase.

10. A system for continuously producing liposomes, comprising:

(a) a first pump for continuously pumping an aqueous phase comprising a phospholipid and at least one target compound;

(b) a second pump for continuously pumping carbon dioxide (CO_2); and

(c) a pressure vessel connected to the first and second pumps, for receiving the aqueous phase and the CO_2, and operable at temperature and pressure conditions above the critical point of CO_2, the pressure vessel having an output valve for continuously releasing an output mixture stream.

11. The system of claim 10 further comprising an inline mixer for mixing the aqueous phase and the CO_2 prior to entering the pressure vessel.

12. The system of claims 9 or 10, wherein the pressure vessel is operated at conditions comprising:

(a) a temperature below about 60° C, preferably about 40° C; and/or

(b) a pressure below about 300 bar, preferably below about 200 bar.

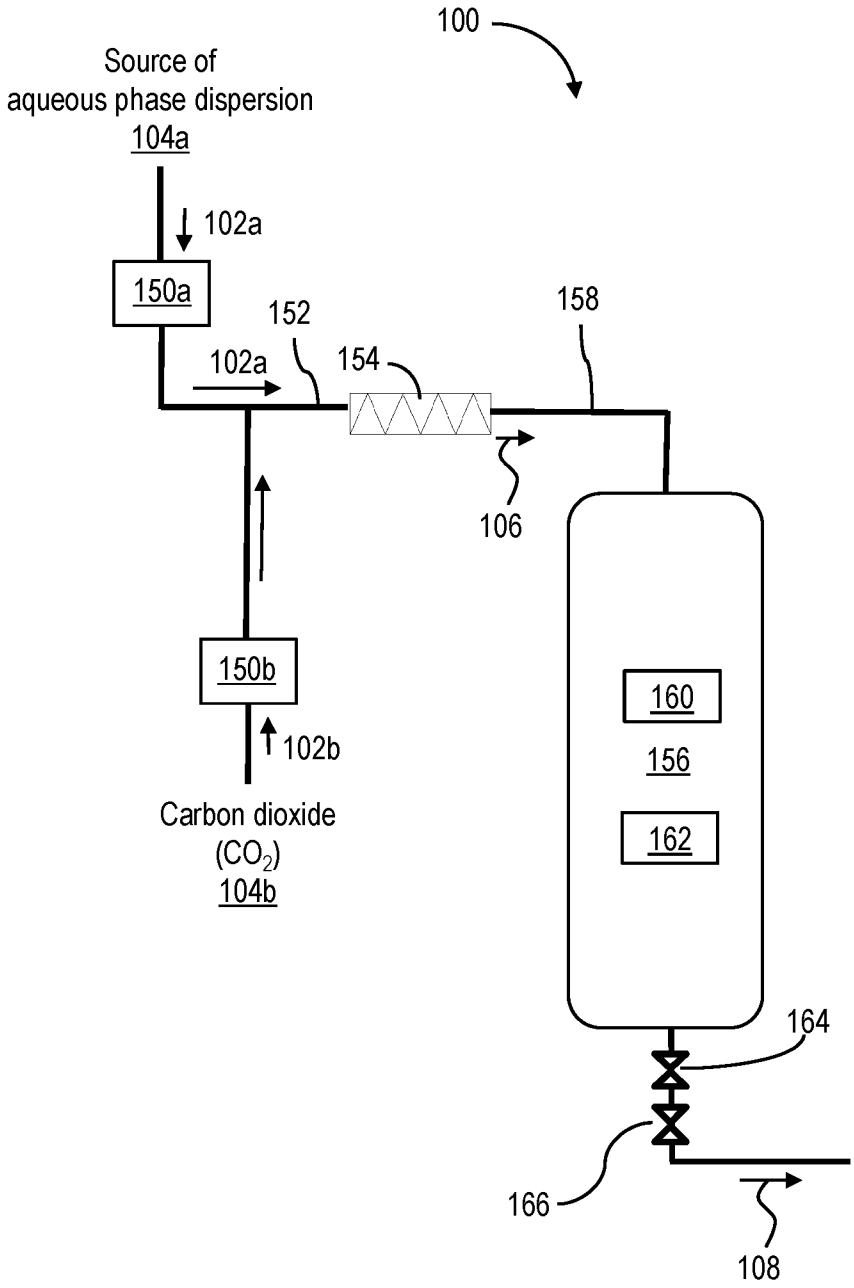
13. The system of any one of claims 10 to 12, wherein the pressure vessel comprises a stirring system.

14. The system of claim 13 wherein the stirring system comprises a paddle or propeller agitator.

15. The system of any one of claims 10 to 14, wherein the pressure vessel is packed with a material to improve the mixing and contact between the substances.

16. The system of claim 15 wherein the packing material comprises glass beads.

17. The system of any one of claims 10 to 16, wherein the system further comprises, prior to the at least one first pump, a heater to apply heat and/or a filter to apply filtration, to the aqueous phase.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Source of
aqueous phase dispersion
104a
102a
150a
152
158
102a
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150b
160
156
162
102b
Carbon dioxide
(CO_2)
104b
164
166
108

FIG. 1

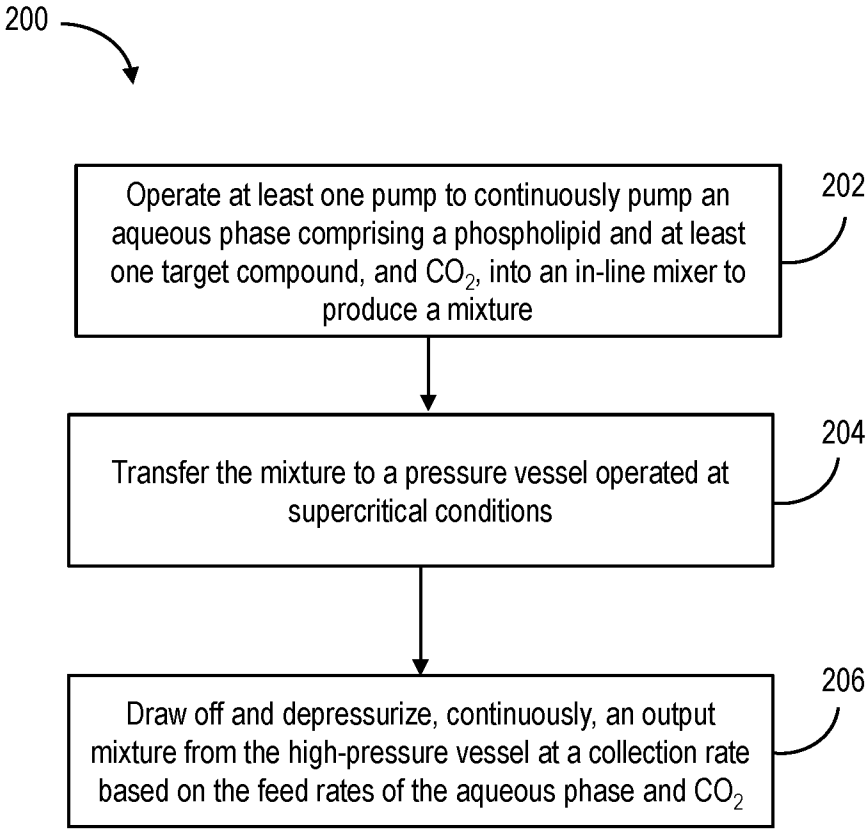
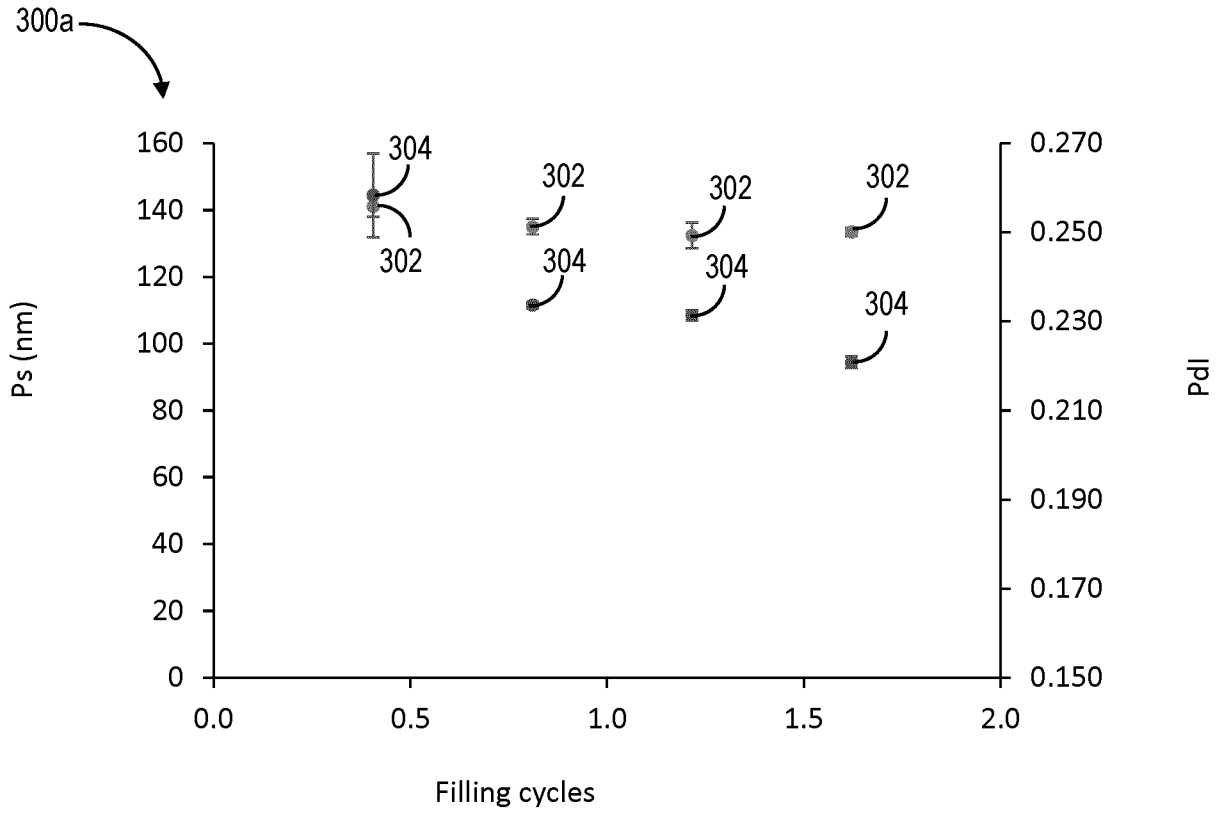

200
Operate at least one pump to continuously pump an aqueous phase comprising a phospholipid and at least one target compound, and CO_2, into an in-line mixer to produce a mixture
202
Transfer the mixture to a pressure vessel operated at supercritical conditions
204
Draw off and depressurize, continuously, an output mixture from the high-pressure vessel at a collection rate based on the feed rates of the aqueous phase and CO_2
206

FIG. 2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160
140
120
100
80
60
40
20
0
Ps (nm)
0.270
0.250
0.230
0.210
0.190
0.170
0.150
PdI
0.0
0.5
1.0
1.5
2.0
Filling cycles
302
304

FIG. 3A

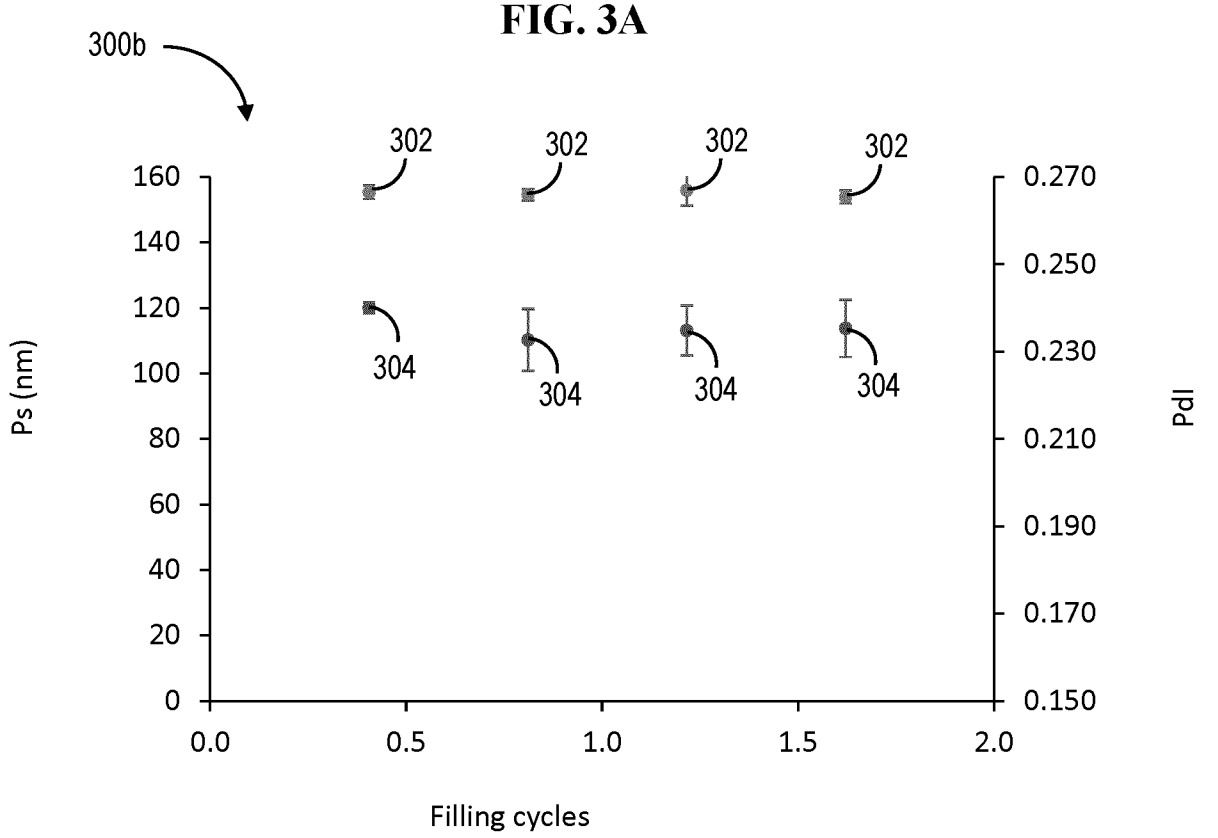
300b
160
140
120
100
80
60
40
20
0
Ps (nm)
0.270
0.250
0.230
0.210
0.190
0.170
0.150
PdI
0.0
0.5
1.0
1.5
2.0
Filling cycles
302
304

FIG. 3B

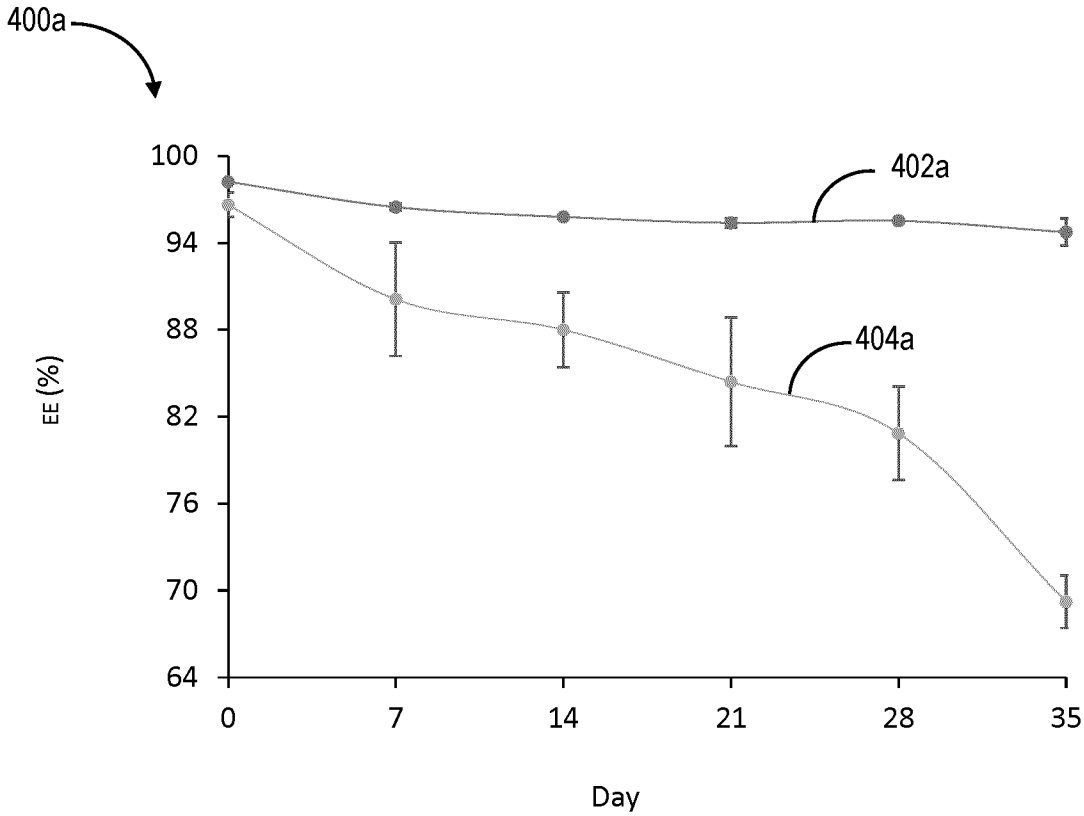
400a
100
94
88
82
76
70
64
EE (%)
0
7
14
21
28
35
Day
402a
404a

FIG. 4A

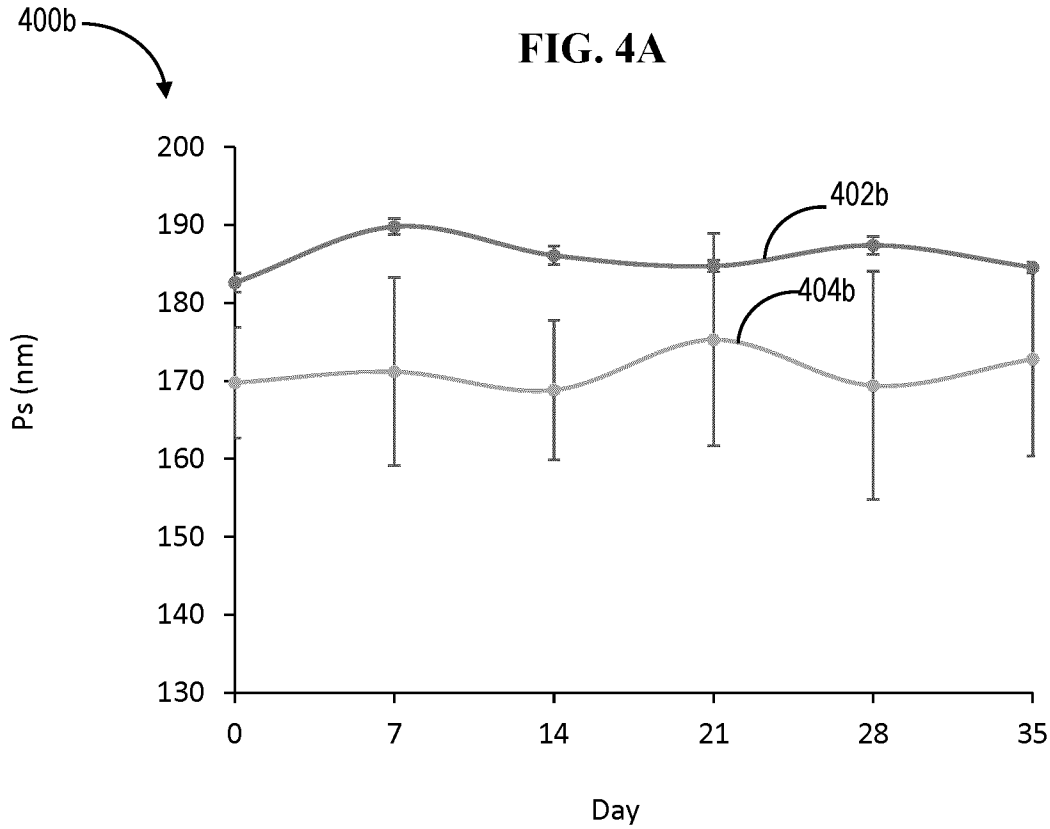
400b
200
190
180
170
160
150
140
130
Ps (nm)
0
7
14
21
28
35
Day
402b
404b

FIG. 4B

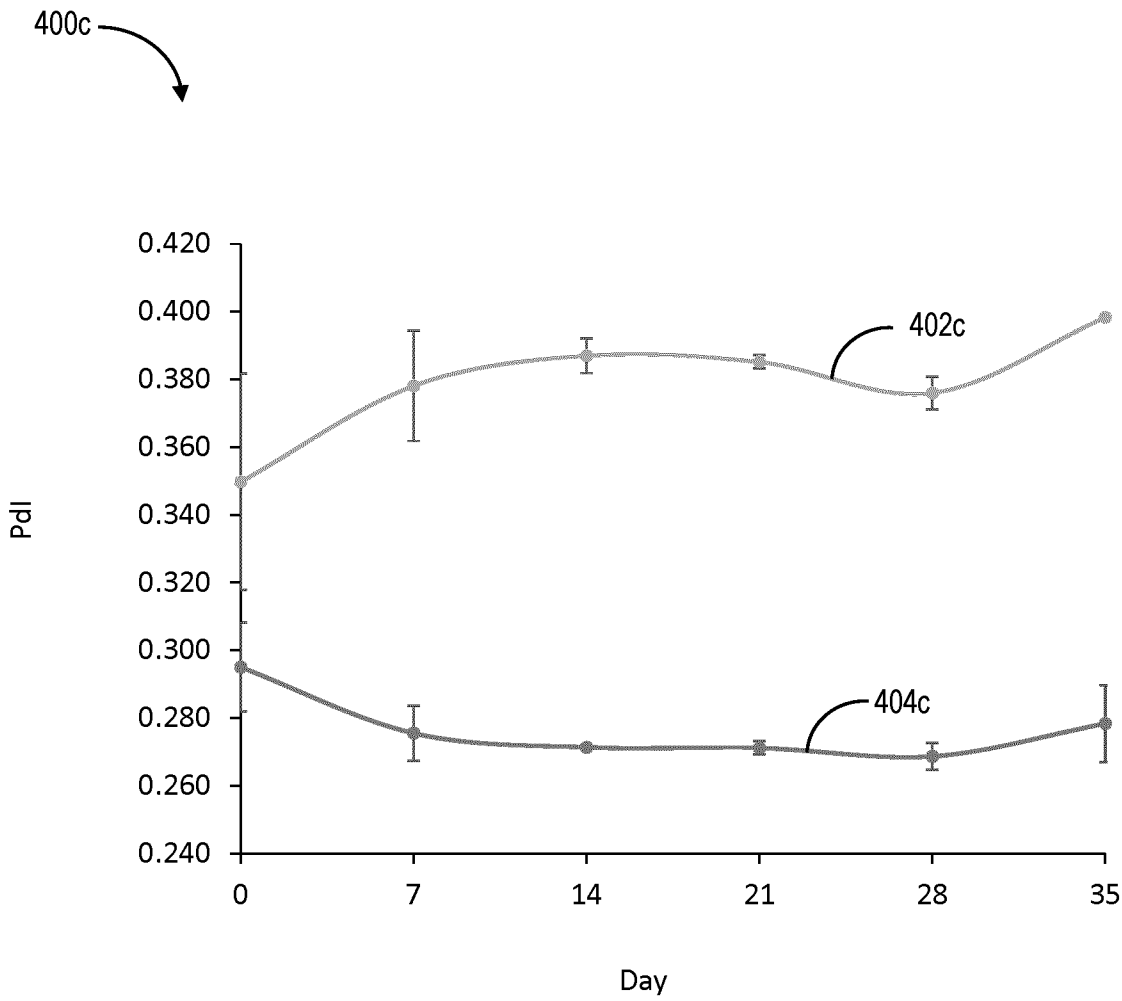
400c
0.420
0.400
0.380
0.360
0.340
0.320
0.300
0.280
0.260
0.240
PdI
402c
404c
0
7
14
21
28
35
Day

FIG. 4C

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2023/051042

A. CLASSIFICATION OF SUBJECT MATTER

IPC: ***B01J 13/02*** (2006.01) , ***B01F 23/00*** (2022.01) , ***B01F 23/41*** (2022.01) , ***B01F 33/71*** (2022.01) , ***B01F 35/75*** (2022.01)

CPC: , B01F 23/043 (2022.01) , B01F 23/414 (2022.01) , B01F 33/71 (2022.01) , B01F 35/7543 (2022.01) , B01J 13/02 (2020.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC: ***B01J 13/02*** (2006.01) , ***B01F 23/00*** (2022.01) , ***B01F 23/41*** (2022.01) , ***B01F 33/71*** (2022.01) , ***B01F 35/75*** (2022.01)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used)

Questel Orbit: liposome*, supercritical, carbon dioxide
Google: liposomes, supercritical, carbon dioxide

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	SANTO, I.E. et al. Liposomes preparation using a supercritical fluid assisted continuous process. Chemical Engineering Journal. 03 April 2014, Vol. 249, pages 153-159, ISSN 1385-8947. **See whole document**	1, 2, 4, 5, 7, 8, 10-12 1-17
X Y	D2: US5776486A (CASTOR et al.) 07 July 1998 (07-07-1998) **Col. 5, lines 19-63; Col. 7, lines 16-35; Fig. 1; Col. 8 line 40-Col. 10, line 43; Table 1; Col. 11, lines 6-8; EXAMPLE 1**	1, 3-5, 7-12, 17 1-17

☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"D"	document cited by the applicant in the international application	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"E"	earlier application or patent but published on or after the international filing date	"&"	document member of the same patent family
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)		
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 27 September 2023 (27-09-2023)	Date of mailing of the international search report 25 October 2023 (25-10-2023)
Name and mailing address of the ISA/CA Canadian Intellectual Property Office Place du Portage I, C114 - 1st Floor, Box PCT 50 Victoria Street Gatineau, Quebec K1A 0C9 Facsimile No.: 819-953-2476	Authorized officer Amanda Livingston (819) 639-8502

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	D3: US2004156911A1 (CHATTOPADHYAY et al.) 12 August 2004 (12-08-2004) **Col. 1, lines 20-28; Col. 2, lines 39-53; Fig. 1; Col. 10, lines 56-66**	6, 15, 16
Y	D4: KARN, P.R. et al. Characterization and stability studies of a novel liposomal cyclosporin A prepared using the supercritical fluid method: comparison with the modified conventional Bangham method. International Journal of Nanomedicine. 21 January 2013. Col. 8, pages 365-377. **Fig. 1, page 367**	6, 13, 14

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		ATE223205T1	15 September 2002 (15-09-2002)
		AU4246296A	17 June 1996 (17-06-1996)
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		CA2205500A1	30 May 1996 (30-05-1996)
		DE69403077D1	12 June 1997 (12-06-1997)
		DE69403077T2	13 November 1997 (13-11-1997)
		DE69528077D1	10 October 2002 (10-10-2002)
		DE69528077T2	17 April 2003 (17-04-2003)
		EP0703778A1	03 April 1996 (03-04-1996)
		EP0703778B1	07 May 1997 (07-05-1997)
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		EP0792143B1	04 September 2002 (04-09-2002)
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		FI115115B	15 March 2005 (15-03-2005)
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		JP4107680B2	25 June 2008 (25-06-2008)
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		NO954756L	26 January 1996 (26-01-1996)
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		WO9615774A1	30 May 1996 (30-05-1996)
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		CA2483563C	26 August 2008 (26-08-2008)
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		CN1318116C	30 May 2007 (30-05-2007)
		DE60310967D1	15 February 2007 (15-02-2007)
		DE60310967T2	21 June 2007 (21-06-2007)
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		EP1549410A1	06 July 2005 (06-07-2005)
		EP1549410A4	16 November 2005 (16-11-2005)
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		EP1590071A2	02 November 2005 (02-11-2005)
		EP1590071A4	01 April 2009 (01-04-2009)
		EP1590071B1	26 September 2012 (26-09-2012)
		ES2280850T3	16 September 2007 (16-09-2007)
		ES2289308T3	01 February 2008 (01-02-2008)
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		JP4421475B2	24 February 2010 (24-02-2010)
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		US2004026319A1	12 February 2004 (12-02-2004)
		US6998051B2	14 February 2006 (14-02-2006)

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		US8642091B2	04 February 2014 (04-02-2014)
		US2006008531A1	12 January 2006 (12-01-2006)
		WO2004004862A1	15 January 2004 (15-01-2004)
		WO2004071634A2	26 August 2004 (26-08-2004)
		WO2004071634A3	07 August 2008 (07-08-2008)
		WO2005016485A1	24 February 2005 (24-02-2005)