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Development of a continuous dense gas process for the production of liposomes

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ABSTRACT

A new process called Continuous Anti-Solvent (CAS) process was developed for the production of liposomes using supercritical CO_2. Unlike the current dense gas technologies, CAS method is a single step and continuous process. Preliminary experiments were conducted in semi-batch mode to determine the most suitable operating conditions (stirring speed = 225 rpm; water/lecithin mass ratio = 21) to ensure an efficient phase mixing in the autoclave. Then, two procedures were developed for the CAS process in the continuous mode. According to the results, the single exit procedure enhances the phase mixing in the autoclave with the formation of a CO_2-in-water emulsion which is a good precursor to liposome formation. Liposomes prepared with the CAS method (P = 9 MPa; T = 308 K; CO_2 flow rate = 0.3 kg h^{-1}; organic solution flow rate = 240 mL h^{-1}; water flow rate = 180 mL h^{-1}) are spherical and multilamellar with a medium diameter included between 10 and 100 μm.

1. Introduction

Phospholipids are natural surfactants which have the ability to form spherical vesicles in presence of water called liposomes. Indeed, phospholipids organize themselves into aqueous phase to reduce the unfavourable interactions between the hydrophilic and hydrophobic parts, leading to one or several phospholipid bilayers encapsulating an inner aqueous phase i.e. liposome structure. Because they are non-toxic and biodegradable, liposomes serve as convenient delivery vehicles for biologically active compounds. As drug delivery systems, liposomes can be administrated either by orally, parenterally or topically way. In order to avoid liposome degradation in human body (phagocytosis, degradation in bile salts and pancreatic lipase, etc.), liposomes are complexed, coated or modified [1,2]. A wider range of applications is possible since the solute can be entrapped either within the phospholipid bilayer (lipophilic compounds), or in the aqueous interior (hydrophilic compounds). The main attribute of liposomes as drug delivery systems is to allow the controlled release of encapsulated drug improving the drug bioavailability or allowing the targeting of drugs to specific tissues. Liposomes are used in a whole range of medical applications such as for the treatment of HIV or other infectious diseases [3–6], in cancer therapy [3,7,8], in gene therapy [3,9,10], in vaccination [3,11,12], in diagnostics [3,13–15], in dermatology [16], in ophthalmology [17]. Liposomes are one of the safest and potentially versatile transfer vectors used to date [18].

Many methods have so far been reported for preparation of liposomes such as Bangham method [19–21], the detergent depletion method [21,22], the ether/ethanol injection method [23–25], the reverse phase evaporation method [25] and the emulsion method [21]. These conventional techniques suffer from some drawbacks linked to scale-up issues and to the frequent use of toxic organic solvents as ethers (isopropyl ether and diethyl ether), methyl alcohol or chlorinated compounds as chloroform [26].

Given the widespread interest in the use of liposomes in medical, pharmaceutical and cosmetic fields, development of new processes using Generally Recognized as Safe (GRAS) solvents such as supercritical CO_2 and in compliance with the constraints imposed by Good Manufacturing Practises (GMP) is required. In these perspectives, dense gas techniques have been developed over the last twenty years [26–46] as alternative solutions to produce liposomes. The use of dense gas and especially supercritical CO_2 enables to employ soft organic solvent such as ethanol and to ensure the easier and quasi-complete removal of the solvent at the end of the process. The use of supercritical CO_2 is of great interest because CO_2 is available (it is a by-product of the chemical industry), non-toxic, non-flammable; and it is supercritical in mild conditions of pressure and temperature. Its critical temperature of 304.1 K allows the treatment of thermosensitive compounds. Lastly, most of its properties can be changed continuously tuning on pressure and/or temperature.

This study focuses on the application of the SAS process to produce liposomes in a continuous way. In the past, several works have been dedicated to high pressure micronization of phospholipids with the SAS process [26,41,45]. Recently, Lesoin *et al.* [47] have hydrated micronized phospholipids powder formed with the SAS

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process to produce liposomes and they have reported the formation of a bimodal population of liposomes, spreading in the range of 0.1–10 μm. But, given that micronized phospholipids react spontaneously upon contact with air [26], micronized material has to be handled with the greatest precautions before the hydration step. The new process affords a solution to this problem because in this process, micronization and hydration are performed in the same autoclave under pressure. This process is called the Continuous Anti-Solvent (CAS) process.

This study is divided in two parts. First of all, a semi-batch mode process has been used to determine the experimental conditions. A second part is dedicated to the study of the process in continuous mode.

2. State of the art in liposome formation using dense gas processes

Generally, dense gas processes of particle generation are classified according to the role of the supercritical fluid in the process. Three different strategies are considered. The compressed CO_2 can be used as a solvent to solubilize the solute, as an anti-solvent to precipitate the solute or as a dispersion agent under critical or subcritical conditions. Concerning our study, works reported in the literature produce either liposomes or proliposomes (finely divided phospholipid particles). The hydration of proliposomes leads to the formation of liposomes.

As concerns works dealing with the production of liposomes, addition of water can be carried out when the system is under pressure or after the depressurization of the system [48].

Castor [27,28], Otake *et al.* [31–35] and Sankar Kadimi *et al.* [38] have presented dense gas processes in which the hydration step is carried out under pressure. In the decompression method presented by Castor [27,28], a mixture composed of lipids, co-solvent (ethanol), hydrophobic drug and water is solubilized with supercritical CO_2 in a high pressure autoclave connected with a second autoclave where the mixture is expanded once solubilization is effective. The supercritical Reverse Phase Evaporation (scRPE) method introduced by Otake *et al.* [31–35] is realized in a single high pressure cell where, phospholipids and co-solvent (ethanol) are first solubilized by supercritical CO_2 and then, water is slowly introduced in the autoclave. Lastly, CO_2 is released and a liposomal suspension is recovered in the cell. Otake *et al.* [37] developed an Improved supercritical Reverse Phase Evaporation (IscRPE) which is a solvent free method. In this method, raw phospholipids and water are introduced in the cell. Then, CO_2 is introduced to reach the desired pressure and after a while, successive depressurizations lead to liposome formation. Sankar Kadimi *et al.* [38] designed a process called the supercritical carbon dioxide mediated process and based on the Gas AntiSolvent method, i.e. CO_2 is used as an anti-solvent to ensure the precipitation of the lipid matter in a batch mode. In this method, the autoclave is first filled with phospholipids solubilized in an organic solvent. Then, supercritical CO_2 is introduced. In a second step, water is introduced and liposomes are formed after depressurization. Thus, these methods differ from the way to put phases into contact. The decompression method produce unilamellar liposomes with a medium size included between 0.01 and 0.3 μm. The scRPE method enables the formation of large unilamellar liposomes with a medium size included between 0.1 and 1.2 μm, while the IscRPE method provides liposomes with a medium size of 1.5 μm. The supercritical carbon dioxide mediated process produces liposomes with diameter ranging from 0.15 to 3 μm.

Castor [27,28], Frederiksen *et al.* [29,30,49] and Meure *et al.* [40] developed dense gas techniques to produce liposomes in which the hydration step was carried out during depressurization. The second method introduced by Castor [27,28] is called the injection method and is the same as the decompression method except that the aqueous phase is introduced in the second autoclave. The first autoclave is used to solubilize a mixture of phospholipids and co-solvent with supercritical CO_2 before the expansion in the second autoclave through a nozzle. Frederiksen *et al.* [29,30,49] developed a technique called the supercritical liposome method and based on the injection method. The experimental set-up is divided in two parts: a high pressure part and an atmospheric pressure part. The first part is dedicated to the solubilization of the phospholipids with supercritical CO_2. Once the solubilization is effective, the mixture is expanded in the second part through the encapsulation capillary where the aqueous phase is brought into contact with phospholipids. Lastly, Meure *et al.* [40] presented a technique called the depressurization of expanded solution into aqueous media (DESAM) in which CO_2 is used as a dispersion agent under sub-critical conditions. Phospholipids are solubilized in an organic solvent and the solution is introduced in the expansion chamber. Then, CO_2 is introduced and the lipid solution is expanded through rapid diffusion of the gas into the solvent. The expansion chamber is connected with a second autoclave filled with a heated aqueous media in which the dispersed mixture is introduced to form liposomes. The injection method produces multilamellar liposomes with a medium size included between 0.06 and 2 μm. The supercritical liposome method produces both small unilamellar liposomes with a medium size included between 0.02 and 0.05 μm and multilamellar liposomes with a medium size of 0.25 μm. The DESAM method enables the formation of small unilamellar liposomes with a medium size included between 0.05 and 0.2 μm.

Dense gas methods producing proliposomes are numerous in the literature. These methods are not specific to the production of liposomes but they are known to be efficient for the comminution of solids difficult to handle. The following processes can be cited: the SAS (Supercritical Anti-Solvent) process [26,41,45,47], the PGSS (Particles from Gas Saturated Solution) [50,51], the ASES (Aerosol Solvent Extraction System) process [42], the GAS (Gas Anti-Solvent) process [38] and the SEDS (Solution Enhanced Dispersion by Supercritical fluids) process [52].

These processes are all batch or semi-continuous processes. Imura *et al.* [32] have first introduced the concept of a continuous dense gas process to form liposomes but this point still remains a challenging task.

3. Materials and methods

3.1. Chemicals

Soy lecithin S100 (94% phosphatidylcholine and 6% others compounds, mainly phospholipids) was purchased from LIPOID (Ludwigshafen, Germany). Analytical grade analysis ethyl alcohol was obtained from Sigma–Aldrich (St Louis, MO). Instrument grade carbon dioxide (purity of 99.7%) from Air Liquide Méditerranée (Vitrolles, France) was used. Double distilled and deionized water was used throughout the experiments.

3.2. Experimental set-up

Fig. 1 shows a scheme of the experimental set-up. It is composed of a stainless steel high pressure autoclave (1) manufactured by New Ways of Analytics (Germany). It can resist to pressures up to 15 MPa and temperatures up to 318 K. Its volume is of 0.763 L and phase visualization is accessible through two borosilicate windows (situated on the autoclave sides). For some experiments, pressures higher than 15 MPa were required and then, a “closed” high pressure autoclave (stainless steel high pressure autoclave,