CONTINUOUS ANTI-SOLVENT METHOD FOR THE PRODUCTION OF LIPOSOMES

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ABSTRACT

A new dense gas process for the formation of liposomes has been developed in our laboratory: the continuous anti-solvent (CAS) method. Experiments were carried out in a stainless steel high pressure vessel with two borosilicate windows. The windows enabled dispersion visualization and the study of the different phases present in the vessel such as water-in-CO_2 or CO_2-in-water emulsions. Liposomes with diameters varying from 0.1 to 100 µm were obtained using raw commercially available soy lecithin. This process is compact, clean and has the major advantage to be continuous compared to the other dense gas processes.

INTRODUCTION

Liposomes, or phospholipid vesicles, are self assembled colloidal particles formed in aqueous solution. In presence of water, interactions between the hydrophilic and hydrophobic parts of phospholipids yield to one or several phospholipid bilayers encapsulating an inner solution phase, i.e. liposome structure. Because they are non-toxic and biodegradable, liposomes serve as convenient delivery vehicles for biologically active compounds. A wider range of applications are possible since the solute can be entrapped either within the phospholipid bilayer, such as for lipophilic compounds, or within the aqueous interior, such as for hydrophilic compounds. Liposomes enable to control the release of encapsulated drug, improving bioavailability, and target the drug to specific tissue. Nowadays, compared with other vectors (polymeric complexes, cyclodextrins, virus and bacteria), liposomes are the safest vectors for active principle delivery in human body. Many methods [1, 2] have so far been reported for preparation of liposomes such as Bangham method [3, 4, 5], the detergent depletion method [5, 6], the ether/ethanol injection method [7, 8, 9], the reverse phase evaporation method [9] and the emulsion method [5]. Those conventional techniques suffer from drawbacks: first of all, several steps are necessary, which are time-consuming and often energy-consuming; secondly they require large quantities of organic solvents which have to be removed at the end of the process; moreover, it may remain solvent traces at the end. To overcome these problems, dense gas techniques have been developed in the last ten years [2, 10 - 27]. Indeed, the use of dense gases, such as supercritical CO_2, has enabled the organic solvent requirement to be reduced compared to conventional techniques. Dense gas technologies can also be considered as more efficient processes for the production of liposomes; for example, adjusting pressure enables to control liposome size distribution. The present study aims to describe a new dense gas process for the formation of liposomes: the continuous anti-solvent (CAS) method which is a single step and continuous process. Before implementing the CAS method, several trials have been carried out in batch mode to determine the most appropriate operating conditions. This preliminary and useful work is also reported in the present article.

MATERIALS AND METHODS

Materials

Soy lecithin S100 (94% phosphatidylcholine + 6% others (mainly phospholipids)) was purchased from LIPOID (Ludwigshafen, Germany). Analytical grade analysis ethyl alcohol was obtained from Carlo Erba (99.8%). Instrument grade carbon dioxide (purity of 99.7%) from Air Liquide Méditerranée (Vitrolles, France) was used. Distilled water was produced in our laboratory. The samples were stored at 4°C. The volume particle size and size distribution of hydrated microparticles were measured by laser diffraction using a Master Sizer S system (Malvern, France). All determinations were carried out triplicate. Stability of the samples was assessed according to several size distribution measurements and visual observation (sedimentation).

Experimental set-up for batch trials

Figure 1 shows a schematic diagram of the experimental set-up which enables to carry out the batch trials. It is composed of a stainless steel high pressure vessel (1) manufactured by New Ways of Analytics (Germany). It can resist to pressures up to 20 MPa and temperatures up to 373 K. Its volume is 0.763 L and melting phase visualization is accessible through two borosilicate windows, situated on the vessel sides. CO_2 (2) is pumped with a liquid high pressure pump (3) (Dosapro Milton Roy, Pont-Saint-Pierre, France) delivering a maximal flow rate of 2.13 $L.h^{-1}$ at 48 MPa. The liquid organic solution (4) injection is performed with a Gilson 307 HPLC pump (5). It delivers a flow rate up to 25 $mL.min^{-1}$ at 60 MPa. Finally, video recordings (6) are taken with a high speed camera Pulnix TM-6CN, with a capture speed up to 25 frames per second with an exposition time of 1/2000 s.

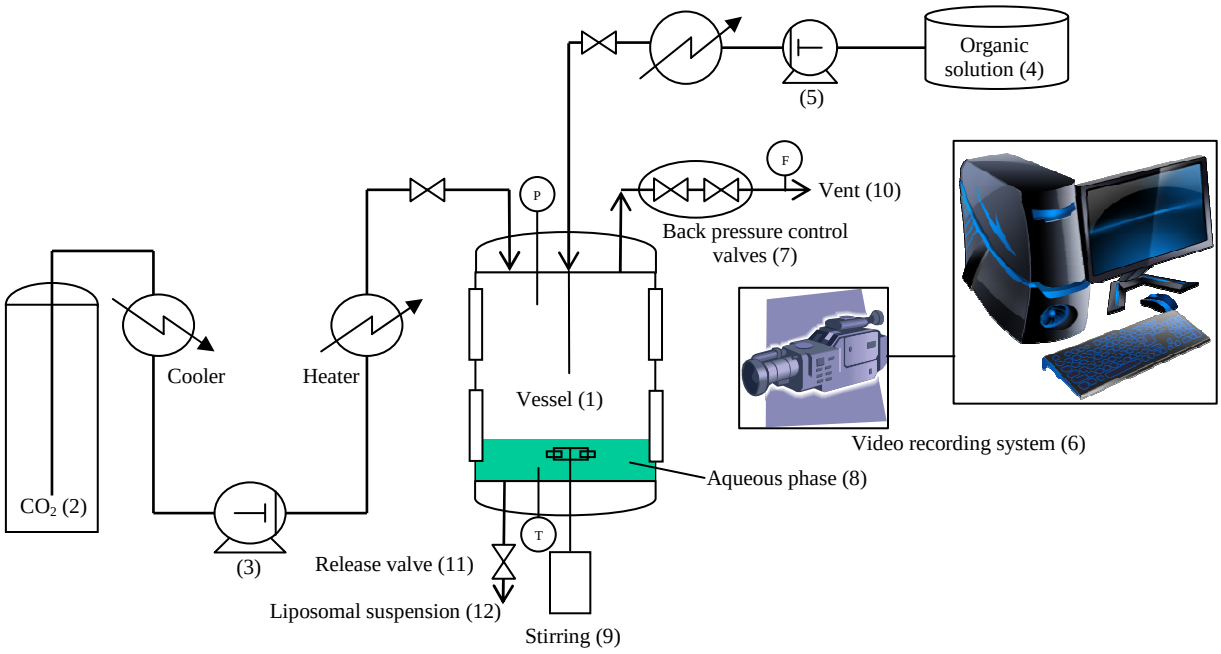

Figure 1 : Experimental set-up to carry out the batch trials

Experimental set-up for the CAS method

The experimental set-up of the CAS method is presented in Figure 2. All materials are the same as for the batch trials except for water that can be injected during the process thanks to a second Gilson 307 HPLC pump (11). Two procedures have been developed with this experimental set-up presented in Figure 2, respectively called CAS1 and CAS2.

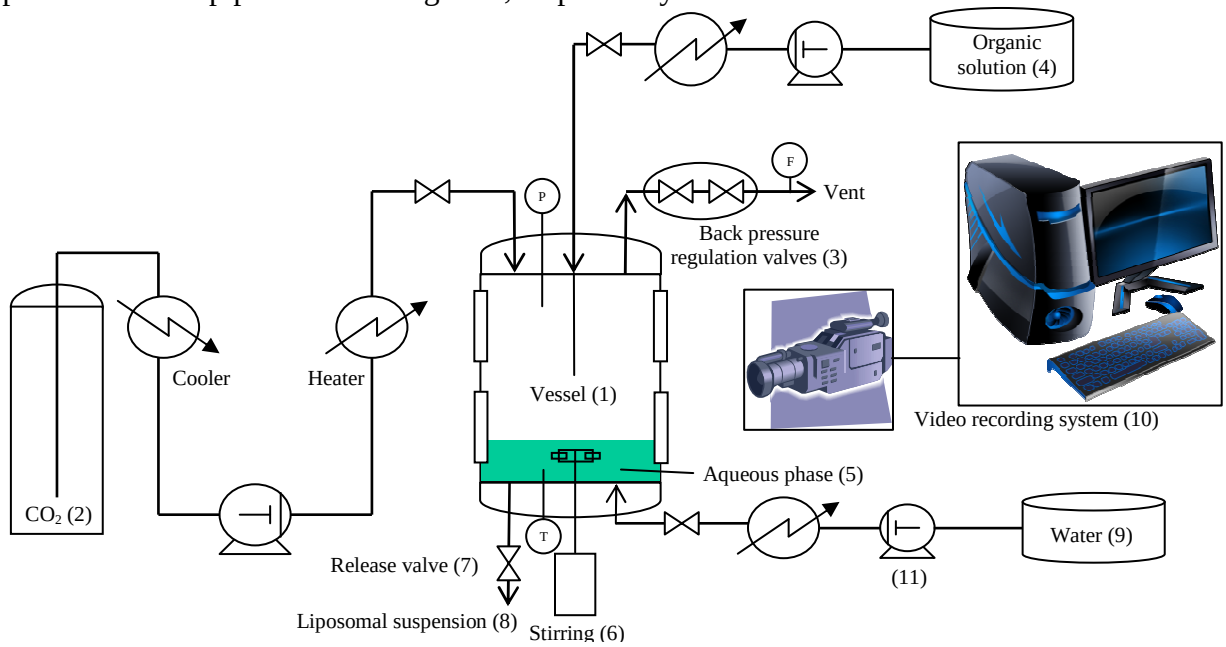

Figure 2 : Schematic diagram of the CAS method experimental set-up: CAS1 and CAS2

Procedures

Batch trials (Figure 1)

First of all, a given amount of water is injected in the vessel (1). Then, the vessel is filled with CO_2 (2) in order to reach the experimental pressure. The vessel is maintained at a constant temperature with the heating system. Once the desired pressure has been set for a given CO_2 flow rate (7), the organic solution (4) containing the solute (soy lecithin) is sprayed (inner capillary diameter 128 µm) into a phase mainly composed of supercritical CO_2. This latter acts as an antisolvent for the solute but as a solvent with respect to the organic solvent. The antisolvent effect of the supercritical fluid entails a supersaturation of the solute into the liquid phase and then its precipitation. Micronized phospholipids fall in the aqueous phase (8) introduced by the bottom of the vessel. The aqueous phase stirring (9) ensures a good mixing and an homogeneous repartition of phospholipids in water. Once a given amount of organic solution has been injected, the CO_2 is released (10) and when the pressure in the vessel has fallen at 2 MPa, the release valve (11) is opened and a liposomal suspension is recovered (12). The video recording (6) enables visualization of the dispersion in the vessel as well as the influence of stirring speed and initial amount of water in the vessel.

All experiments are carried out at 308 K, with a CO_2 flow rate of 750-800 $g.h^{-1}$, a lecithin concentration in the organic solution of 15wt%, an organic solution flow rate of 4 $mL.min^{-1}$, and absolute ethanol as solvent. The experimental conditions employed for the other parameters (pressure, stirring speed, initial composition of water versus CO_2 and final concentration of lecithin in water) are summarized in Table 1. All experiments are undertaken twice to ensure reproducibility.

Table 1 : Range of operating conditions (batch trials)

Set	Pressure / MPa	Stirring speed / rpm	Initial composition of water versus CO_2 / wt%	Final concentration of lecithin in water / $g.mL^{-1}$
1	9	100	33.37	0.040
2	9	225	33.37	0.040
3	9	300	33.37	0.040
4	9	225	26.98	0.057
5	9	225	26.98	0.040
6	9	225	39.46	0.036
7	12	225	26.98	0.057

CAS methods

As explained before, two procedures have been developed for the CAS method. In the first case, CAS1, the positioning of CO_2 exit differs from the liposomal suspension one, as shown in Figure 2. In this case, the set-up has two exits. In the second case, CAS2, a same exit is used for the CO_2 and the liposomal suspension (by the bottom of the vessel as shown in Figure 2 at the release valve level). Thus, the system is a single exit process.

In CAS1, an initial amount of water is injected in the vessel (1); then, the vessel is filled with CO_2 (2) and maintained at constant temperature. The CO_2 flow rate is set (3) and the organic solution (4) is sprayed. In the vessel, the liquid solution (5) is stirred (6) during the whole process. After a while - when the solution at the bottom of the vessel becomes homogeneous and whitish - the release valve (7) is slowly opened, while injection of the organic solution is kept constant. Thus, liposomal suspension (8) is continuously extracted from the bottom of the vessel during the steady state of the process. According to the flow of liposomal suspension, water (9) is injected to maintain a constant liquid volume in the vessel.
In CAS2, contrary to the first case, the vessel (1) remains closed during the batch state. The organic solution (4) is sprayed and the liquid phase (5) is stirred (6). When the liquid phase is homogeneous and whitish, the back pressure regulation valves (3) are slowly opened to yield the desired CO_2 flow rate. Then, CO_2 and liposomal suspension are continuously extracted from the vessel by the same exit (7) during the steady state.

Table 2 : Range of operating conditions (CAS1 method)

Set	Initial composition of water versus CO_2 / wt%	*Lecithin wt% in organic solution*	*Molar ratio CO_2/EtOH*	*Stirring speed* / rpm	*Water injection flow rate* $mL.min^{-1}$	m_{water} *injected during the process* / g	$T_{transitory\ regime}$ / min	$T_{steady\ state}$ / min
8	30.94	15	4.8	300	3	71.83	5	54
9	30.94	15	4.7	300	3	117.88	5	59
10	30.94	15	5.1	300	3	135.37	5	58
11	30.94	15	4.8	300	8	180.47	5	55
12	17.59	12	4.6	300	2	69.42	16	47
13	14.28	13	4.6	300	2	88.30	8	58
14	14.39	13	4.6	300	2	68.33	6	51
15	13.65	20	5	300	2	84.19	7	60
16	14.12	25	5.2	300	2	82.66	8	58

The video recording (10) enables visualization of the dispersion, water-in-CO_2 and CO_2-in-water emulsion formation and destabilization, and also of the level of the liquid solution at the bottom of the vessel which indicates the steady state settlement. Several trials have been

carried out to find the working point; especially the initial amount of water and the injection water flow rate have been adjusted. Thanks to the results of these trials, the time necessary to reach the steady state is estimated.

All experiments are carried out at 9 MPa and 308 K, with an organic solution flow rate of 4 $mL.min^{-1}$ and absolute ethanol as solvent. The variation ranges of the other parameters for CAS1 are detailed in Table 2 and in Table 3 for the CAS2.

Table 3 : Range of operating conditions (CAS2 method): reproducibility study

Set	Initial composition of water versus CO_2 / wt%	*Lecithin wt% in organic solution*	*Molar ratio CO_2/EtOH*	*Stirring speed* / rpm	*Water injection flow rate* $mL.min^{-1}$	m_{water} *injected during the process* / g	$T_{transitory\ regime}$ / min	$T_{steady\ state}$ / min
17	29.66	15	1.78	300	3	33.70	10	60
18	29.66	15	1.78	300	3	18.61	10	20
19	29.03	15	1.78	300	4	45.92	10	20

RESULTS

Batch process

The aim of this section is to present the results of trials carried out with the batch process set-up and to show how these results allowed choosing the experimental parameters of the CAS method. As presented in Table 1, pressure, stirring speed and initial composition of water versus CO_2 are the three process variables whose influence is studied upon phase visualization in the vessel and liposome size distribution curves.

Pressure effect

Figure 3 shows the particle size distribution of liposomes obtained at 9 and 12 MPa. The pressure may have an effect. The smallest liposomes are obtained at the highest pressure (more precisely pressure drop).

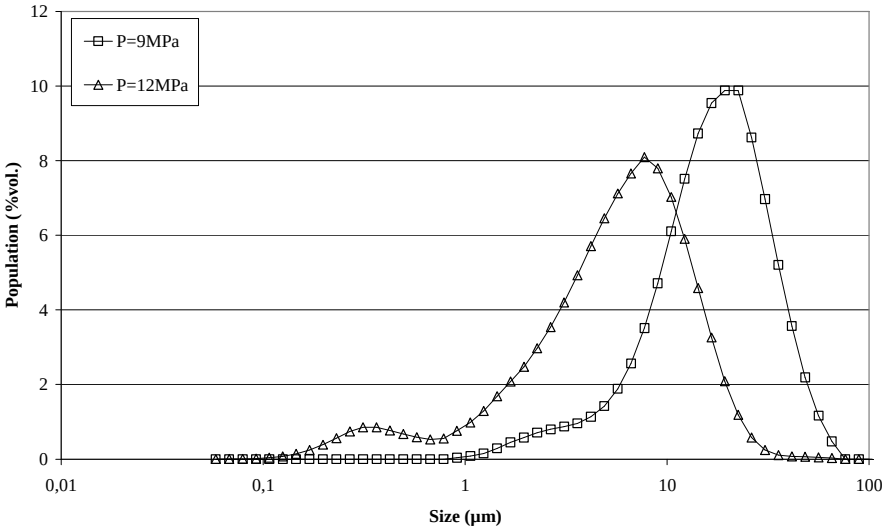

Figure 3: Particle size distribution of liposomes: *P*=9 MPa corresponds to set 4 and *P*=12 MPa corresponds to set 7 in Table 1

Influence of the stirring speed

According to Figure 4, a stirring speed of 300 rpm is necessary to form a homogeneous mixture in the vessel.

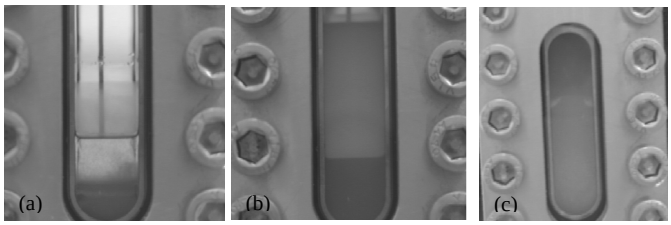

Figure 4: Photographs of the emulsion in the high pressure vessel during the injection of the organic solution: three different stirring speeds were tested: (a) 100 rpm (set 1). (b) 225 rpm (set 2). (c) 300 rpm (set 3).

Influence of the initial composition of water in the vessel

In order to study the influence of the initial composition of water (against CO_2), two types of trials have been carried out. First of all, the same quantity of lecithin is injected whatever the initial composition of water (varying lecithin concentration in water); and secondly, injected lecithin quantity is ajusted to maintain the same concentration of lecithin in water. Three compositions of water were tested: 26.98wt%, 33.37wt% and 39.46wt%. Figure 5 shows the three levels of water in the high pressure vessel, and Figure 6 presents the size distribution curves of liposomal suspension formed. According to Figure 6, for the constant final concentration of lecithin in water, a composition of 33.37wt% leads to the formation of liposomes with a smaller size distribution (mean diameter between 0.1 and 1 µm for 33.37wt%, compared with 1 and 10 µm for 26.98wt%). So, this value of composition will the choosen one for the initial composition of water in the vessel.

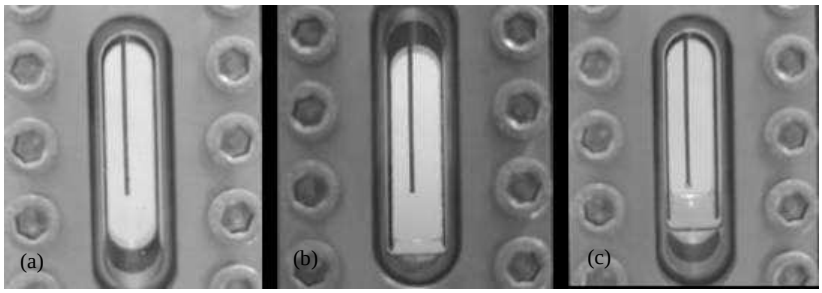

Figure 5 : Photographs of the vessel for three different initial composition of water (versus CO_2): (a) 26.98wt% (set 4). (b) 33.37wt% (set 2). (c) 39.46wt (set 6).

Thus, the value of 33.37wt% versus CO_2 for the initial composition of water in the vessel will be tested for the continuous anti-solvent method in the next part.

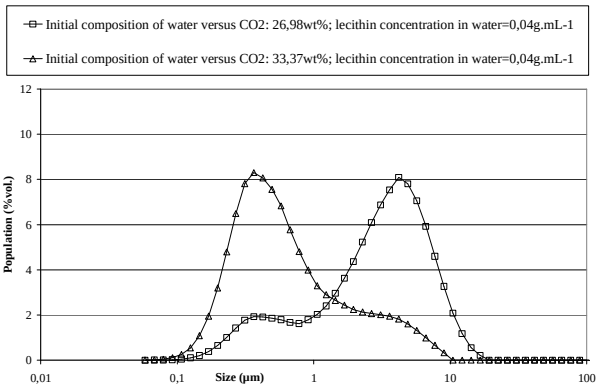

Figure 6 : Size distribution curves of liposomes according to the initial composition of water (wt% versus CO_2) in the vessel: two initial compositions of water and the same final lecithin concentration in water: 26.98% (set 5) and 33.37% (set 2)

Liposome size distributions

The average polydispersity of the CAS liposome samples ranged from 0.1 to 100 µm. With CAS1, most of the size distribution curves are bimodal, since they are unimodal with CAS2. Figure 7 shows that the amount of water injected during the continuous state of the process greatly influences liposome particle size distribution for CAS1. It appears that the fraction of biggest liposomes (above 10 µm) increases when the amount of water injected increases.

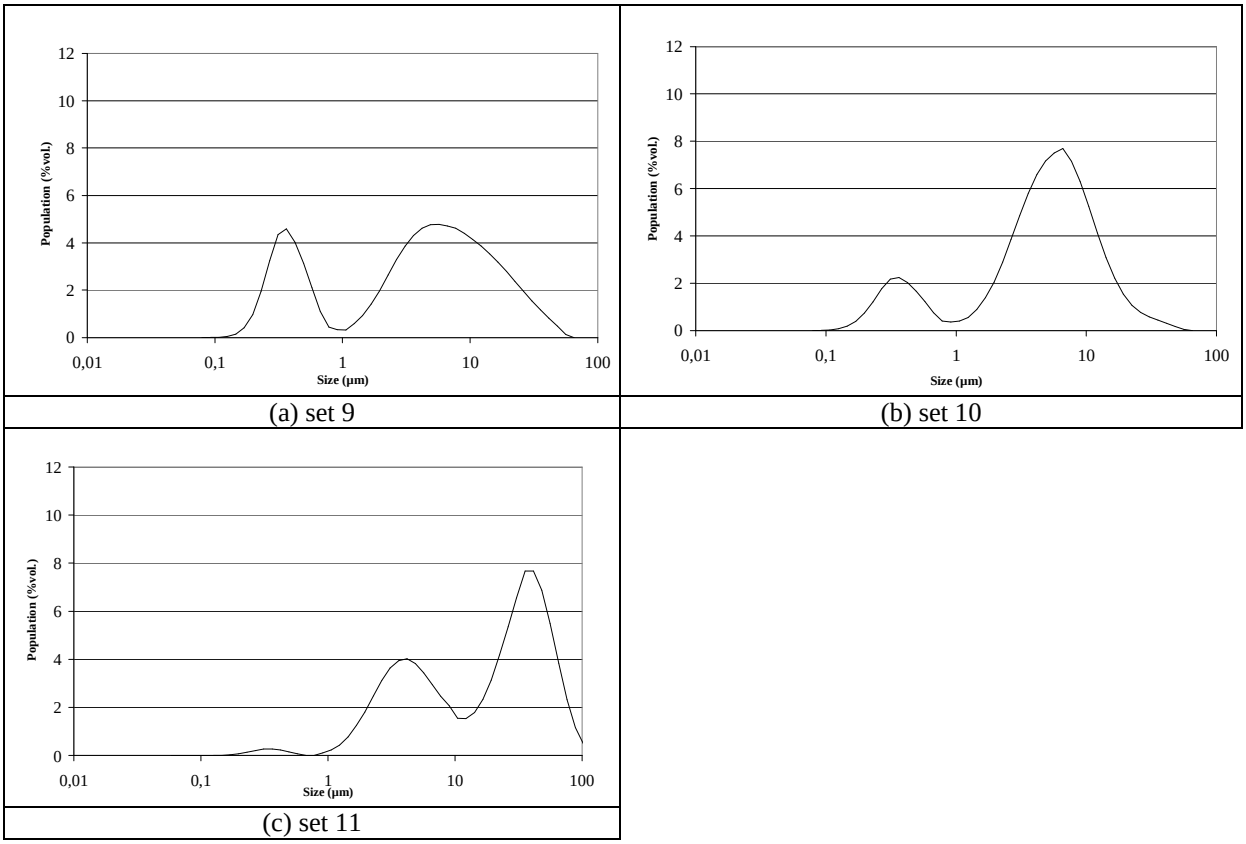

Figure7: Population of liposomes (%vol.) versus size of liposomes (µm) respectively for set 9 (a), set 10 (b) and set 11 (c)- *Influence of the amount of water injected during the continuous step*

According to Figure 8, runs 12, 13, 14, 15 and 16 are not meaningful. One may conclude that the weight fraction of lecithin in the organic solution exhibits minimal effect. Concerning experiments undertaken for CAS1, the biggest volume fraction (76%) of liposomes with diameter below 1 µm is achieved for run 12. Run 12 seems to represent the most interesting experimental conditions (P = 9 MPa, T = 308K, initial composition of water versus CO_2 = 17.59wt%, lecithin concentration in the organic solution = 12wt%, Organic solution flow rate = 4 mL.min^{-1}, CO_2/solvent molar ratio = 4.6, stirring speed = 300 rpm, quantity of water added during the continuous step = 69.42 g) employed for CAS1, which correspond to the smallest amount of water injected during the trial.

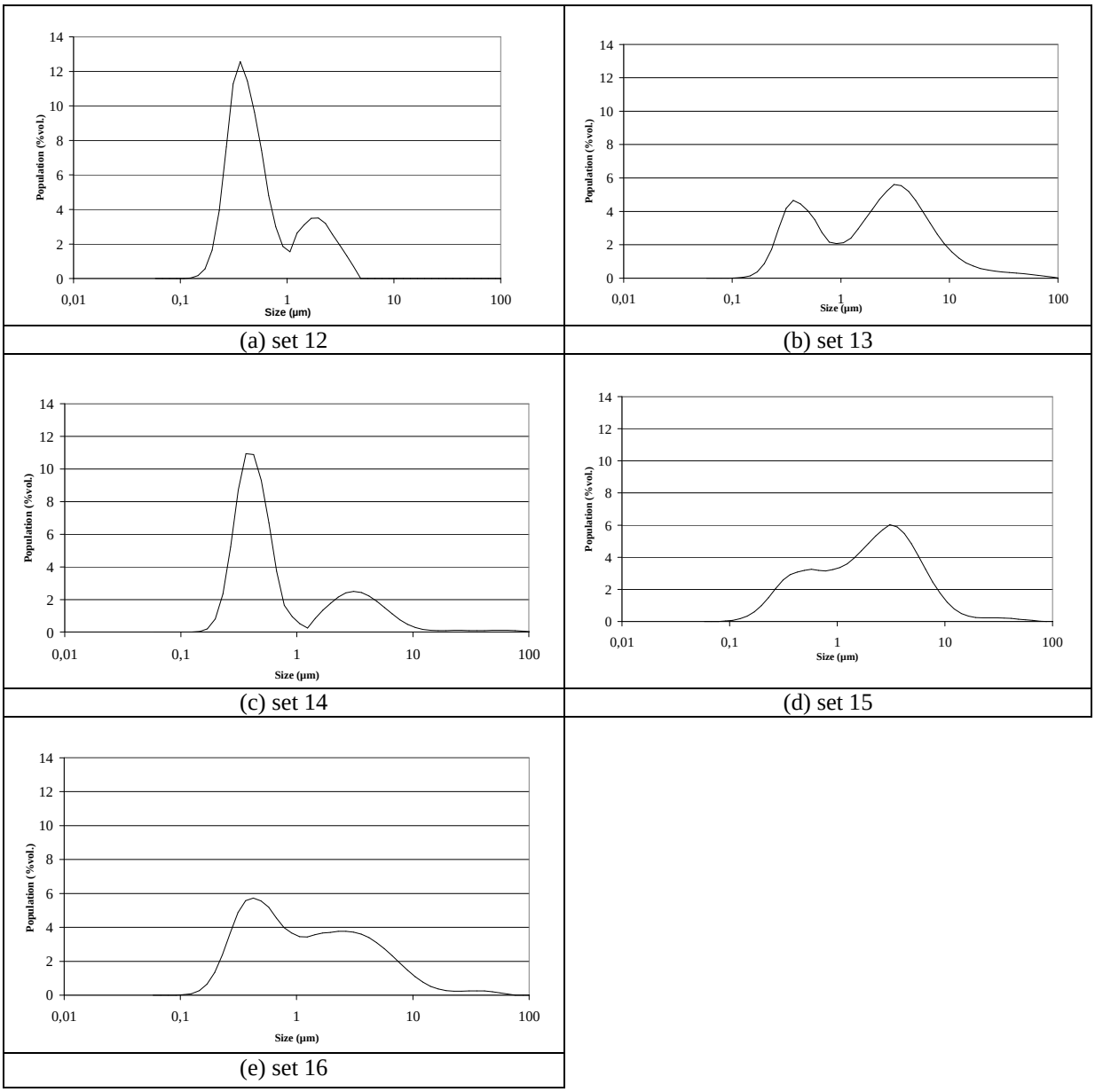

Figure 8 : Population of liposomes (%vol.) versus size of liposomes (µm) respectively for set 12 (a), set 13 (b), set 14 (c), set 15 (d) and set 16 (e)-*Influence of the lecithin concentration in the organic solution*

Contrary to CAS1, CAS2 enables the formation of liposomes with unimodal size distribution mainly centred between 10 and 100 µm (Figure 9); and reproducibility is

validated. Then, it can be concluded that CAS2 process gives promising results. Tuning on experimental conditions as pressure drop, the liposome size may probably be reduced.

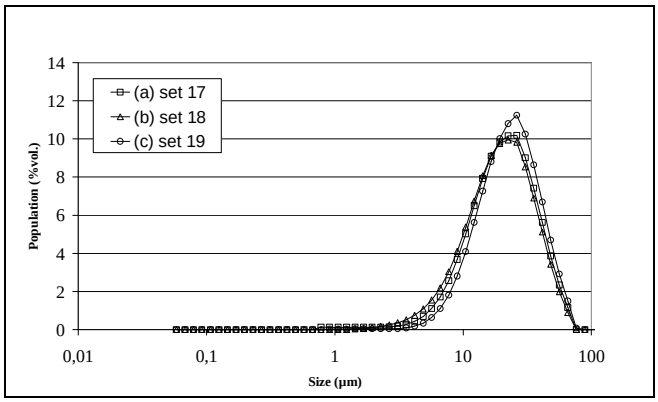

Figure 9 : Population of liposomes (%vol.) versus size of liposomes (µm)

CONCLUSION

This study developed a new dense gas process for the production of liposomes. This method has the main advantages to use less toxic solvent, to be a single step method and a continuous process. Meaningful details about the trials carried out in the batch mode are exposed. Trials in batch mode have enabled to find the experimental conditions for the continuous mode. Two procedures were tested for the continuous mode: CAS1 and CAS2. Video recordings enabled to follow the evolution of the mixture in the vessel. CAS2 seems to offer the most efficient mixing conditions of the phases. The quantity of water added during the continuous step appears to play a major role for CAS1. Results achieved for CAS2 are promising since unimodal size distributions were obtained for the formed liposomes.

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