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Gas To Liquid Ratio Optimization For The Reduction Of Ethanol Residue For The Production Of Liposomes With A Supercritical Assisted Technique

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ABSTRACT

Liposomes are vesicles made of an external double layer of phospholipids and an internal aqueous core. Conventional production methods generally work in batch mode; this causes difficult replication of sample production and the presence of huge amount of solvent residue such as ethanol or chloroform. SuperLip (Supercritical assisted Liposomes formation) is a novel process developed for the 1-shot production of nanoliposomes. This process was developed on lab scale and already validated on a great variety of compounds.

In this work, an optimization of an important operative parameter, the Gas to Liquid Ratio (GLR), is presented for the first time. The results obtained in this work were very important to understand process mechanisms. Indeed, changing the Gas To Liquid Ratio (GLR), it was possible to switch from micrometric to nanometric mean dimensions of vesicles and also to control the amount of ethanol residue in the final suspension. Particularly, by increasing GLR, nanometric liposomes were obtained with a lower content of ethanol. This result could be used for the commercialization of liposome-based pharmaceutical products, in which the content of solvent residue has to be controlled according to regulations.

Keywords

Liposomes, drug carriers, solvent residue

Abbreviation list

PC: L-α-Phosphatidylcholine, **GLR**: Gas to Liquid Ratio, **EE**: Encapsulation Efficiency

SR: Solvent Residue, **MD**: Mean Diameters, **SD**: Standard Deviation

INTRODUCTION

The production of drug carriers has found great applications in the pharmaceutical [1] and cosmetic field [2] for many decades. Drug carriers are spherical objects that generally provide some benefits over traditional drug formulations [3], because they can easily protect the entrapped drug from degradation and premature metabolism during their release in the human body [4].

Many processes were developed for the production of drug carriers on laboratory, pilot and industrial scale [5], and in many occasions organic solvents were involved. Often, huge quantities of solvent were found in the core of the produced drug carriers; as a consequence of this, the applicability of those carriers was compromised and considered unacceptable for food and pharmaceutical products [6].

Among drug carriers, liposomes are spherical vesicles characterized by a inner aqueous core and an external double lipidic layer. Since their discovery by Dr. Alec Bangham in 1965, liposomes were recognized to be marketable products [7]. However, conventional liposomes production methods involved the use of different kind of organic solvents, such as methanol, ethanol and chloroform. Among the solvents used for the production of vesicles, ethanol belongs to ICH class 3, less toxic than ICH class 2 solvents such as chloroform, generally used for the thin layer hydration method [8].

Supercritical assisted techniques merged as successful alternatives to traditional methods in liposomes production [9]. Those innovative techniques have been developed to try to reduce the amount of organic solvents found in the suspensions, exploiting the solubility power of carbon dioxide above the critical point [10]. Despite the success of these first attempts, a difficult control of particle size distribution, low encapsulation efficiencies and solvent residues were still a great challenge for liposomes production.

For this reason, a dense gas process called Supercritical assisted Liposome Formation (SuperLip) was developed for the production of lipid vesicles. It managed to guarantee a better control of particle size distribution with reproducible nanometric dimensions and high encapsulation efficiencies of proteins such as bovine serum albumin [11], dyes as fluorescein [12], antibiotics as theophylline [13] and antioxidants as eugenol and lipoic acid [14].

The aim of this work is to optimize the Gas to Liquid Ratio (GLR) to reduce the amount of ethanol involved in the system and consequently into collected liposomes suspension. First, empty liposomes will be produced to study the effect of GLR on liposomes mean dimensions, polydispersion index and ethanol residue. Once optimized the GLR, two model compounds will be entrapped into liposomes using the best operating conditions: vancomycin [15, 16], a hydrophilic antibiotic entrapped in the inner core of liposomes, and farnesol [17] in the double lipidic layer of liposomes, since it is a lipophilic compound.

MATERIALS AND METHODS

Materials

Phosphatidylcholine from egg yolk (PC) was supplied in powder form by Sigma-Aldrich (99%). The organic solvent, ethanol, chosen for lipids dissolution was obtained by Sigma-Aldrich (≥99.8%). Carbon dioxide (CO_2) was purchased by Morlando Group, Italy (>99.4% pure). For the production of the aqueous compartment of liposomes, distilled water was self-produced in our laboratories using a distillatory. Farnesol (sesquiterpene antioxidant) and vancomycin (ocular antibiotic) were provided by Sigma-Aldrich in powder form. For all the performed experiments, the materials were used as received.

Apparatus

Supercritical assisted Liposomes formation (SuperLip) was developed on lab-scale layout. Two feed lines, sub-critical carbon dioxide (through Ecoflow pump, mod. LDC-M-2, Lewa, Germany) and ethanol+lipid solution (through high pressure precision pump, Model 305, Gilson, France) were provided from the top of the stainless steel formation vessel. The two feed were mixed in a stainless steel saturator, thermally heated at the temperature of 40 °C. The obtained Expanded Liquid (EL) was fed to the high pressure formation vessel. An aqueous solution was pumped in the formation vessel through continuous atomization with a 80 μm nozzle for droplet creation. Water droplets were covered by lipids in the formation vessel, while a bulk, located at the bottom of the vessel, was used to stabilize vesicles. The organic solvent was removed from the top of the formation vessel through a decompression step that eliminated the expanded liquid, separating carbon dioxide from ethanol. Carbon dioxide flow rate was controlled with a rotameter (mod. N.5-2500, Serval 115022, ASA, Italy) and liposomes were collected from the bottom of the formation vessel in liquid suspension.

Methods

Liposome mean dimensions and zeta potential were measured with Dynamic Light Scattering (Mod. Zetasizer Nano S, Worcestershire, UK).
Liposome morphology was observed with a Field Emission-Scanning Electron Microscope (FE-SEM, model LEO 1525, Carl Zeiss SMT AG, Oberkochen, Germany). A drop of liposome suspension was spread on aluminum stub and left drying at room temperature for 2-3 days. Dry liposome suspension was then covered with gold to make the sample conducible through a sputter coater (thickness of 250 Å, model B7341, Agar Scientific, Stansted, U.K.).
In the case of vancomycin and farnesol loaded liposomes, not entrapped drug was detected from the supernatant after sample centrifugation [18]. The absorbance measurement were performed at the wavelength of 230 nm for vancomycin while it was 260 nm in the case of farnesol absorbance detection, using an UV-Vis spectrophotometer. The encapsulation efficiency was calculated using the following equation:

$$EE\ [\%] = \frac{ppm_{theoretical} - ppm_{supernatant}}{ppm_{theoretical}} 100$$

In this equation, $ppm_{theoretical}$ is the amount of compound dissolved in the water phase, while $ppm_{supernatant}$ represents the not entrapped drug present in the supernatant. Measurements were performed in triplicates and all the data were shown as mean value ± standard deviation.
Ethanol residue in the final liposomes suspensions was also analyzed, to monitor the efficiency of solvent removal from the aqueous bulk after the separation of the expanded liquid. The solvent residue was measured using a head space sampler (mod. 50 Scan; Hewlett & Packard, Palo Alto, California) coupled to a Gas Chromatograph interfaced with a Flame Ionization Detector (GC-FID; mod. 6890 Agilent Series; Agilent Technologies Inc., Wilmington, Delaware). Ethanol was separated using a fused-silica capillary column of 50 m length, 0.25 mm internal diameter, 0.40 µm film thickness (mod. DB-WAX; Agilent, United States). GC oven temperature was maintained at 40 °C for 8 min. The injector was maintained at 180 °C (split mode, ratio 1:1) and helium was used as the carrier gas (1 mL/min). Head space conditions were: equilibration time 60 min at 100 °C, pressurization time 2 min and loop fill time 1 min. Head space samples were prepared in 10 mL vials filled with 2 mL of suspension. Analyses were performed on each sample in three replicates.

RESULTS

The idea at the base of this work was to optimize the Gas to Liquid Ratio parameter. In particular, its effect on final solvent residue and Particle Size Distributions was studied. Empty liposomes were produced varying the ethanol flow rate from 10 mL/min to 1.9 mL/min, fixing carbon dioxide flow rate at 6.5 g/min. As a consequence of this, the Gas to Liquid Ratio was varied from 0.44 to 6.00. Lipid concentration in ethanol was set at 5 mg/mL, while operative pressure was fixed to 100 bar for all the experiments. Temperature in the formation vessel was fixed to 40 °C. The nozzle chosen for water atomization has a diameter of 80 µm. Soon after the SuperLip process, the samples were post-treated using evaporation under vacuum to eliminate solvent residue. This operation was performed at 40 °C and 150 rpm for 20 minutes.

Table 1. Mean Diameters (MD), Standard Deviations (SD), Polydispersion Index (PDI), Solvent Residue (SR) of empty liposomes with Gas To Liquid Ratio in the range from 0.44 to 6.00

Gas To Liquid Ratio	**Ethanol Flow rate**	**System composition**			**Mean diameter**	**Solvent Residue**
	[mL/min]	**CO_2**	**C_2H_5OH**	**H_2O**	**MD [nm] ± SD**	**[ppm]**
0.44	10	0.21	0.47	0.30	1697 ± 679	4102
1.50	9	0.39	0.24	0.35	940 ± 263	3023
3.83	3.5	0.46	0.11	0.42	155 ± 71	2450
6.00	1.9	0.47	0.06	0.45	149 ± 72	1890

As shown in **Table 1**, by increasing the Gas to Liquid Ratio, nanometric liposomes with a mean diameter included between 149 ± 72 nm and 155 ± 71 nm were produced. Instead, working at GLR less than 1.50, it was discovered that liposomes of micrometric and sub-micrometric dimensions were produced, with mean dimensions between 940 ± 263 nm and 1697 ± 679 nm. It is possible to affirm that the GLR parameter has a significant affection on the control of liposome size and particle size distributions. Instead, increasing the GLR, solvent residue decreased from 4102 ppm for a gas to liquid ratio of 0.44 down to 1890 ppm for a GLR of 6.00. As a conclusion on the first section of this work, the main observation is that the best optimized conditions were achieved

working with a GLR of 6.00, since it was possible to produce nanometric liposomes with the lowest solvent residue.

In the second part of this work, hydrophilic compound vancomycin was entrapped in the inner core of liposomes with theoretical loadings of 15 % on lipid mass base. The same theoretical loading was repeated for the entrapment of a lipophilic compound, farnesol. Gas To Liquid Ratio was fixed at 6.00 as optimized with the previous set of experiments. Mean Diameter, Encapsulation Efficiencies and Solvent Residue of these experiments were reported in **Table 2**.

Table 2. Mean Diameter (MD), Encapsulation Efficiency (EE) and Solvent Residue of vancomycin and farnesol loaded liposomes with Gas to Liquid Ratio of 6.00

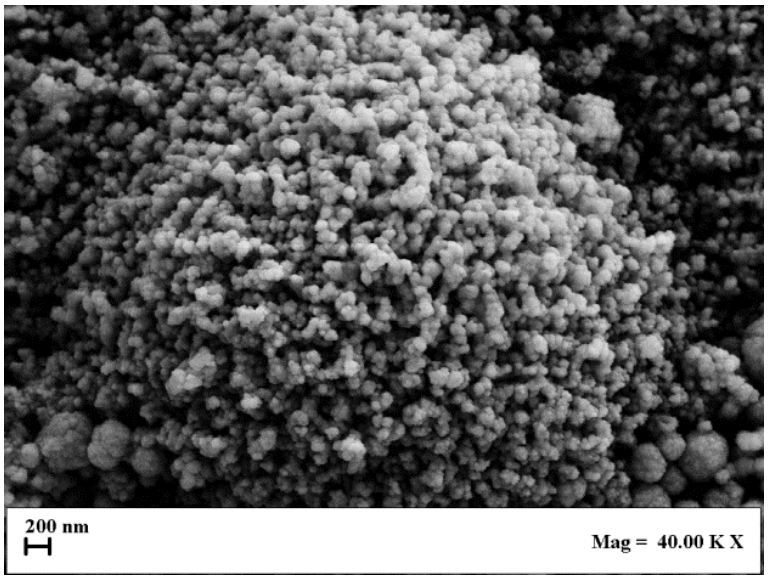
Compound	**Theoretical Loading [%]**	**Mean Diameter MD [nm] ± SD**	**Encapsulation Efficiency [%]**	**Solvent Residue [ppm]**
Vancomycin (hydrophilic)	15	180 ± 48	60.0 ± 0.8	10
Farnesol (lipophilic)		126 ± 35	74.0 ± 1.1	1680

Looking at **Table 2**, mean diameters of vancomycin and farnesol loaded liposomes are included between a minimum of 126 ± 35 nm and a maximum of 180 ± 48 nm. It is confirmed that working at GLR equal to 6.00, it is possible to control particle size distribution of liposomes and to create nanometric vesicles. The encapsulation efficiency was high up to 76.7 ± 1.9 % for vancomycin loaded liposomes, and up to 74.0 ± 1.1 % for farnesol loaded liposomes.

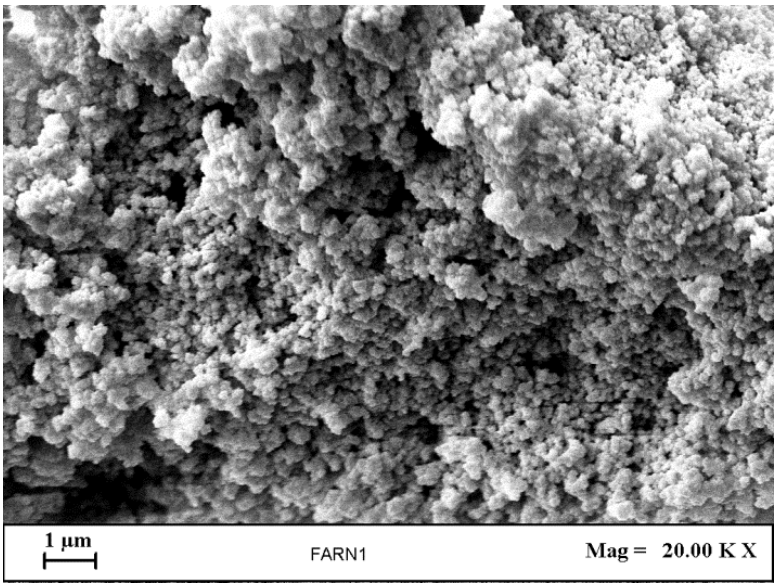
After evaporation under vacuum, solvent residue of vancomycin loaded liposomes was 10 ppm for 15 % loaded vesicles. Comparing these results with empty liposomes, ethanol residue was much lower. In fact, empty liposomes had a solvent residue of 1890 ppm for a GLR equal to 6.00. Instead, for farnesol loaded liposomes, solvent residue was 1680 ppm for 15 % loaded samples.

Lipophilic loaded liposomes had a solvent residue more similar to empty liposomes. It is possible that the affinity between ethanol and water caused the entrapment of a huge amount of organic solvent in the inner core of liposomes. Instead, in the case of vancomycin loaded liposomes, the hydrophilic compound contributed to remove organic solvent from water droplets during formation process. In this manner, the ethanol was rejected to the external bulk of liposomes suspension, leaving 10 of the organic solvent in the inner core of liposomes.

Field Emission-Scanning Electron Microscope (FE-SEM) images were obtained. **Figure 1a** refers to vancomycin loaded liposomes while **Figure 1b** refers to farnesol loaded liposomes.



(a)



(b)

Figure 1. Scanning Electron Microscope of vancomycin and farnesol loaded liposomes produced with GLR 6.00

Figure 1a showed liposomes loaded with 15 % w/w vancomycin on lipid mass base. Nanometric mean dimensions were confirmed, according to **Table 2.** The shape of the vesicle was round and the surface was smooth.

A second observation was performed on farnesol loaded liposomes (**Figure 1b**). Also in this second case, vesicles mean dimensions were confirmed; but, more aggregation phenomena were shown. This was probably due to the higher affinity of farnesol with ethanol in the lipidic structure.

CONCLUSIONS

Working with a Gas to Liquid Ratio (GLR) above 1.50 produced nanometric liposomes (mean diameter of about 100 nm), while under that limit, the obtained vesicles were micrometric (mean diameter of about 2 µm). As a consequence of that, it was possible to tune liposomes mean dimensions by tuning the Gas to Liquid Ratio, according to the kind of application.

A second result that was observed was that solvent residue decreased by decreasing the Gas to Liquid Ratio on empty liposomes, from 4567 ppm to 1890 ppm. Moreover, this was also confirmed by the entrapment of two model compounds into liposomes: vancomycin (among 134 and 10 ppm) and farnesol (among 4939 and 1680 ppm).

The chosen hydrophilic compound better rejected ethanol content from the inner core of liposomes, obtaining down to 10 ppm of solvent residue inside the core of the vesicles. Lipophilic compound did not respond to tests as expected, because of its affinity with ethanol.

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