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Operative Parameters Optimization Production of Liposomes for the Encapsulation of Hydrophilic Compounds Using a New Supercritical Process

Paolo Trucillo, Roberta Campardelli*, Ernesto Reverchon

Department of Industrial Engineering, University of Salerno, Via Giovanni Paolo II, 132, 84084, Fisciano, SA), Italy
rcampardelli@unisa.it

Liposomes are spherical vesicles formed by a inner aqueous core and a double lipidic layer around it. Conventional techniques for the production of liposomes are characterized by several drawbacks, like the production of micrometric vesicles, a difficult control of the Particle Size Distribution (PSD) and low encapsulation efficiencies (EE) of hydrophilic compounds. Many supercritical semi-continuous techniques were proposed in literature. They are successful in the intent of producing liposomes of smaller diameter, but the EE of hydrophilic compounds and the reproducibility are still a challenge.
For this reason, it was recently proposed a new supercritical process whose aim is to invert the steps of production of liposomes, by first creating water droplets and then to fast surround them by phospholipids. We discovered that the high diffusion coefficient of phospholipids in supercritical carbon dioxide allows a fast coverage of water droplets preserving the drug content into the liposome core. In this work, hydrophilic compounds were encapsulated in the vesicles produced using SuperLip, such as Fluorescein, Bovine Serum Albumin (BSA) and Ampicillin, obtaining monodispersed spherical vesicles with a mean size from 100 to 300 nm. Operative parameters like water flow rate and lipid to water mass ratio were optimized. The EEs were evaluated with UV-Vis spectroscopy according to methods reported in literature, and obtaining high values up to 99 % for the three investigated compounds.

1. Introduction

Liposomes are nanometric and sub-micrometric vesicles made of an internal aqueous core and an external lipidic double layer. In an aqueous medium, phospholipids spontaneously self-assemble forming spherical closed structures (Liu, 2016). Hydrophilic compounds can be encapsulated in the water core while lipophilic compounds can be entrapped in the lipidic layer. Liposomes are powerful drug delivery systems since their barrier is similar to natural cell walls (Meure et al., 2008); most vesicles formulations are not toxic and biodegradable. Moreover, lipidic barrier can prevent drugs from degradation phenomena, like the effect of light and heat. The production of liposomes smaller than 200 nm gives them the possibility to circulate in the human blood without being digested by macrophages before they can reach target tissues (Cho et al., 2008). Tumor tissues are also characterized by nanometric interstices; this makes nanosomes able to accumulate inside them and release anticancer drugs.
For all these reasons, liposomes are widely studied, especially for the huge applications in which they can be used: medicine, food, agriculture, cosmetics (Fakhravar, 2016) and gene therapy (Li et al, 2015). Several liposomes preparation methods have been developed, but most of them suffer of many limitations like batch operations, low encapsulation efficiency of hydrophilic compounds, difficult removal of organic solvents and bad control of Particle Size Distributions. Supercritical fluid have been used as powerful promoters for biomedical applications (De Marco, I., 2016); moreover, supercritical assisted techniques for the production of liposomes have been proposed in literature to overcome these drawbacks (Lesoin et al., 2011), but the Encapsulation Efficiency (EE) of hydrophilic compounds are still low.
A novel supercritical technique named SuperLip (Supercritical Assisted Liposome Formation) has been proposed in literature (Espirito Santo et al., 2014). The process gives an original solution to the problems

presented; water droplets are first generated and then they are covered by phospholipids. Liposomes samples produced in this manner are monodispersed, free of organic solvent and with high EE of hydrophilic drugs.
For these reasons, SuperLip has been used in this work to encapsulate hydrophilic compounds of different kind: ampicillin, an antibiotic used for ophthalmic therapeutic applications; fluorescein, a hydrophilic dye used to detect biochemical signals; bovine serum albumin, a model protein of high molecular weight. The effects of water flow rate variation were detected on those samples, as well as the variation of lipidic concentration fed to the system.

2. Materials, Apparatus and Methods

2.1 Materials

L-α-phosphatidylcholine lyophilized powder from egg yolk (PC, purity ~ 60 %) was purchased by Sigma Aldrich, Milan, Italy. Being purchased by Sigma Aldrich (Milan, Italy), Ethanol was used as a solvent for lipids (EtOH, purity > 99.8 %). Carbon dioxide was provided by SON, Naples, Italy (CO_2, purity > 99.4 %). Distilled water was produced in our laboratories. Ampicillin (A, purity > 98 %), Fluorescein (F, purity > 99 %) and Bovine Serum Albumin (BSA, purity > 96 %) were purchased by Sigma Aldrich, Milan, Italy.

2.2 Apparatus

Supercritical Carbon Dioxide (sc-CO_2) and ethanol+lipids were fed separately in a thermally heated saturator (S_1) where an expanded liquid (EL) was obtained. Then, the EL was fed to a high pressure stainless steel formation vessel (FV). Another line was fed to the FV to generate water droplets containing the hydrophilic drug. The atomization was obtained using a nozzle of the diameter of 80 μm. CO_2 was pumped using an Ecoflow pump (mod. LDC-M-2, Lewa, Germany). Ethanol solution was prepared with PC, while water solution was obtained dissolving in water ampicillin, fluorescein and BSA in different experiment sets. Water and ethanol solutions were pumped using two separated high pressure precision pumps (Model 305, Gilson, France). Ethanol solution was fed at the flow rate of 3.5 mL/min. CO_2 was fed to the saturator together with ethanol solution at Gas to Liquid Ratio (GLR) equal to 2.4, on mass base. Pressure and temperature were set at 100 bar and 40 °C. Water flow rate was first fixed to 10 mL/min and then to 0.70 mL/min. A decompression step (S_2) was used to separate CO_2 and ethanol using a stainless-steel separator, operating at 10 bar and 25 °C. The scheme of the presented process is drawn in Figure 1.

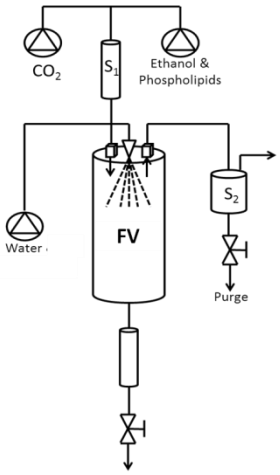

Figure 1: SuperLip plant scheme

2.3 Methods

Liposome suspensions were characterized using Dynamic Light Scattering (DLS) instrument (Mod. Zetasizer Nano S, Worcestershire, UK), to measure mean size (MD), polydispersion index (PDI) and standard deviation (SD) of the vesicles. This instrument works at 25 °C and is equipped with a 5.0 mW He-Ne laser operating at 633 nm with a scattering angle of 173°. Three measurements of the same sample were performed and values reported are the mean over three measurements.
Liposomes morphology was analysed by FE-SEM (LEO 1525, Carl Zeiss SMT AG). Samples were centrifuged, then their supernatants were eliminated and the vesicles were re-suspended in distilled water.

This operation was repeated twice. Finally, a droplet of liposome suspension was set over an aluminium stub and dried at air for 2 days. The samples were then coated with a gold layer.
The determination of EE of liposomes was measured indirectly from the supernatant method (Otake et al, 2006); it was performed in triplicates and the resulting value is the average EE. To obtain an accurate estimation of the drug entrapped, liposomes suspensions were ultra-centrifuged at 13000 rpm for 30 min at -4 °C. Then the amount of drug in the supernatant was measured using a Micro-volume UV-Vis spectrophotometer (BioSpec-nano, Shimadzu Scientific Instruments, Columbia, USA) at the wave length of 515 nm for fluorescein (Campardelli et al, 2016), at 280 nm for bovine serum albumin and at 225 nm for ampicillin. The Encapsulation Efficiency was calculated as the complement to 100 of the percentage of drug present in the supernatant, as expressed mathematically in the Eq(1).

$$EE\ [\%] = 100 * \left(1 - \frac{\mathrm{ppm_{loaded}}}{\mathrm{ppm_{supernatant}}}\right) \quad (1)$$

Results of Eq(1) are listed in the Tables 1, 2, 3, 4. Fluorescein drug release tests were performed at 37 °C using again UV-Vis spectrophotometer at the wave length of 515 nm. The drug profiles were determined in 250 mL of distilled water continuously stirred at 200 rpm in absence of light. Drug release tests were performed in triplicates and the curves proposed in the results are the mean profile obtained.

3. Results

In this work, the effect of the variation of some process parameters was studied. Liposomes were prepared with 5 mg/mL lipid concentration; vesicles were loaded with 1, 3 and 6 % ampicillin in mass ratio with lipids. Water flow rate was set to 0.70 mL/min.
At these conditions, a stable liposome suspension was obtained with a single step of production if compared with traditional methods. Mean diameters (MD), PDI and Encapsulation Efficiencies are reported in Table 1.

Table 1: MD, PDI and EE of liposomes loaded with 1, 3 and 6 % w/w of ampicillin

Test	Ampicillin Theoretical Loading [w/w, %]	MD [nm] ± SD	PDI	EE [%]
1	1	213.3 ± 20.2	0.19	62.6
2	3	380.5 ± 30.4	0.16	77.5
3	6	385.5 ± 42.4	0.22	92.5

Observing Table 1, the mean value of liposomes produced was increased by increasing ampicillin theoretical loading. In fact, liposomes loaded with 1 % w/w had a mean diameter of 213.3 ± 20.2 nm. With a 3 % w/w loading, the PSD was moved to higher values, with a mean diameter of 380.5 ± 30.4 nm. With a 6 % w/w theoretical loading a further increase in the mean diameter up to 385.5 ± 42.4 nm was obtained.
Regarding the EE, 1 % w/w loaded liposomes had an EE of 62.6 %, while for 3 % w/w and 6 % w/w loaded liposomes the EE was increased to 77.5 % and 92.5 % respectively. The increased theoretical loading was effective on the EE increase. This probably happened because the more quantity of drug was dissolved into the initial water solution, the more was entrapped in the water droplets. Indeed, droplets coverage speed was higher than drug diffusion from internal core to external bulk.
As a further proof of the versatility of SuperLip, 1 % w/w fluorescein was loaded into liposomes to study the effect of lipid concentration variation. In this new set of experiments, 500 mg, 750 mg and 1000 mg of PC were dissolved in 100 mL of ethanol to obtain 5, 7.5 and 10 mg/mL lipid concentration. The only modified working parameter was water flow rate, which was set to 10 mL/min. Since the quantity of water fed to the system was fixed, the only variation was given by the PC mass. That made the PC/Water ratio to change. Mean diameters, PDI and Encapsulation Efficiencies are reported in Table 2 and Figure 2.

Table 2: MD, PDI and EE of liposomes loaded with 1% w/w of fluorescein at 10 mL/min water flow rate

Test	Lipid Concentration [mg/mL]	PC/H_2O	MD [nm] ± SD	PDI	EE [%]
4	5	2	204.4 ± 38.8	0.38	87.5
5	7.5	3	188.7 ± 37.7	0.40	76.5
6	10	4	160.3 ± 32.1	0.40	87.9

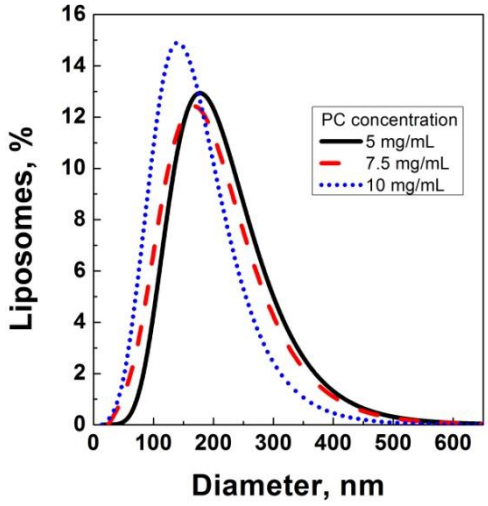

Figure 2: Frequency distributions of 1 % w/w fluorescein loaded liposomes with water flow rate of 10 mL/min

As shown in Table 2 and Figure 1, fluorescein did not significantly modify Particle Size Distributions because mean diameters were smaller than 205 nm. By increasing the PC mass used to prepare the samples, a little decrease of mean diameters was observed. The sample prepared with 5 mg/mL of PC had a diameter of 204.4 ± 38.8 nm while the sample with maximum lipid concentration had a mean diameter of 160.3 ± 32.1 nm. The measured EE was between 76.5 and 87.9 % without a significant trend, since it was not affected by PC concentration.

The same set of experiments for the production of fluorescein loaded liposomes was performed with a smaller water flow rate, 0.70 mL/min. In this case, PC/Water ratio was furthermore modified because PC and water mass fed to the system were both changed. In Table 3 the mean diameters, PDI and EE of this set of experiments were reported.

Table 3: MD, PDI and EE of liposomes loaded with 1 % w/w fluorescein at 0.70 mL/min water flow rate

Test	Lipid Concentration [mg/mL]	PC/H_2O	MD [nm] ± SD	PDI	EE [%]
7	5	25	476.5 ± 127.7	0.54	96.4
8	7.5	38	448.3 ± 132.5	0.59	99.9
9	10	50	912.3 ± 329.8	0.72	99.4

As seen in Table 3, by increasing lipidic concentration, a progressive increase of average liposomes dimensions was observed, from a mean diameter of 476.5 ± 127.7 nm to 912.3 ± 329.8 nm for the 10 mg/mL lipidic concentration. This effect was mainly due to the formation of more lipidic double layers around water droplets. This phenomenon was highly evident with this kind of compound and especially for a reduced water flow rate. The reduced speed of water droplets traduced into a major residence time of water droplet in the formation vessel, giving lipids the time to produce a higher number of layers.

Moreover, mean dimensions of liposomes were higher than the set produced at 10 mL/min also because with a lower water flow rate, fluid velocity at the exit of water injector is reduced. This caused a minor phenomenon of jet break up and the formation of bigger droplets. The probable presence of a higher number of lipidic layers gave the possibility to entrap further more quantity of fluorescein in the inner core, from 96.4 % to 99.9 %, higher than the EE of the previous fluorescein samples. Since the water speed is decreased, a major number of water droplets is covered by phospholipids before the drug diffuses into the external bulk.

To complete the study of fluorescein encapsulation efficiency, drug release experiments were performed in vitro at 37 °C on samples loaded with 1 % w/w fluorescein and 5, 7.5 and 10 mg/mL lipid concentration (0.70 mL/min water flow rate), comparing them with free fluorescein drug release. The kinetics curves were compared in Figure 3.

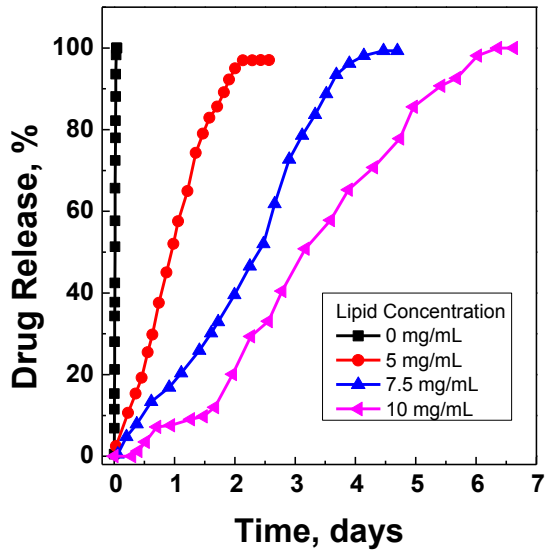

Figure 3: Drug release comparison: free fluorescein, 5 mg/mL PC, 7.5 mg/mL PC, 10 mg/mL PC

As shown in Figure 3, free fluorescein was totally released after 2 hours. Liposomes prepared with 5 mg/mL lipid concentration reached the maximum values after 2 days, the sample with 7.5 mg/mL reached it after 5 days and the most loaded one released all its content after 7 days almost. What was evident is the delaying effect on drug release, due to the presence of a higher lipidic content. This results confirmed the hypothesis that a greater amount of PC caused the formation of a greater number of lipidic double layers, making the vesicles more compact and giving the fluorescein more obstacles to overcome during diffusion into the external bulk.

To conclude this work, bovine serum albumin (BSA) was encapsulated into liposomes. Ampicillin is a hydrophilic protein with a higher molecular weight compared to the previous studied compounds. Indeed, BSA was chosen to demonstrate that it is possible to encapsulate also molecules bigger than ampicillin and fluorescein, using SuperLip. The water flow rate was set to 10 mL/min and the other process parameters were not changed. A set of experiments was performed by varying BSA theoretical loading from 10 % w/w to 30 % w/w and then to 60 % w/w in ratio with PC mass. In this case PC/Water ratio did not change because water flow rate and lipid concentration were not modified. Results are reported in Table 4.

Table 4: MD, PDI and EE of liposomes loaded with 10, 20 and 30 % w/w bovine serum albumin

Test	BSA Theoretical Loading [w/w, %]	PC/H_2O	MD [nm] ± SD	PDI	EE [%]
10	10	2	123.0 ± 12.3	0.20	62.5
11	30	2	144.7 ± 16.0	0.22	83.5
12	60	2	244.6 ± 36.7	0.30	93.9

By increasing drug loading (Table 4), liposome mean diameter increased. The increased mean diameter was ascribed to the increased viscosity of a higher BSA concentration in water solution. The PDI were also increased by increasing theoretical loading, because the PSDs become polydispersed. The EE was increased from 62.5 % for 10 % w/w loaded sample to 93.9 % for 60 % w/w loaded sample, because the high viscosity of water solution slows drug diffusion to the external bulk.

A FE-SEM image of 10 % w/w BSA loaded liposomes is reported in Figure 4.

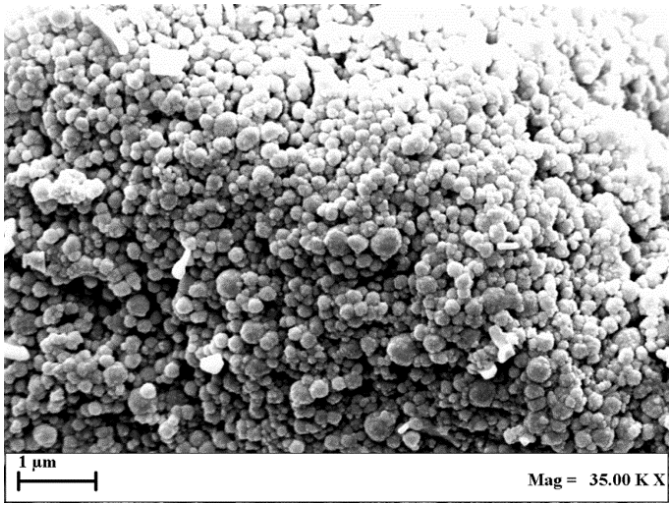

Figure 4: FESEM image obtained from 10 % w/w BSA loaded liposomes

From Figure 4, it is possible to see that liposomes appear spherical with dimensions greatly smaller than the reference bar of 1 µm, confirming the granulometric data reported up.

4. Conclusions and perspectives

Ampicillin, fluorescein and BSA encapsulation experiments were performed with SuperLip by varying some process parameters. Increasing Ampicillin theoretical loading, nanometric and stable liposomes were produced with high EE. Increasing the lipidic concentration, liposomes mean diameters were not modified by higher water flow rates while they significantly increased at lower water flow rates. Indeed, the higher lipidic concentration caused the formation of a major number of lipidic layers that showed a delaying effect on fluorescein drug release. EE were still high up to 99.9 %. These good results were also confirmed for BSA loaded liposomes, that become smaller by increasing drug theoretical loading.
In the future, other families of hydrophilic and lipophilic compounds will be encapsulated in liposomes using SuperLip, with the intent of studying the molecular interaction between active principles and lipids. The variation of other process parameters such as the GLR and the temperature will also be investigated.

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