

A Versatile Supercritical Assisted Process For The Production of Nanosomes: Development, Production And Commercialization

P. Trucillo, R. Campardelli*, E. Reverchon

Department of Industrial Engineering, University of Salerno, Via Giovanni Paolo II, 132 – 84084 Fisciano (SA), ITALY

www.supercriticalfluidgroup.unisa.it, www.nanotechliposomes.com

[*rcampardelli@unisa.it](mailto:rcampardelli@unisa.it)

ABSTRACT

Liposomes are spherical vesicles characterized by a double layer of phospholipids and an inner water volume. They are generally employed as biocompatible drug carriers used for the vehiculation of hydrophilic or lipophilic compounds. Conventional methods for the production of liposomes suffer of many drawbacks such as low encapsulation efficiencies, difficult control of particle size distribution and toxicity due to the presence of the solvent residue. Moreover, those techniques generally need expensive and time-consuming post-processing steps.

To overcome limitations linked to liposome production, a novel process named SuperLip (Supercritical assisted Liposome formation) has been developed. The key of the process is the inversion of the formation steps during liposome production. Droplets of water are first created using a nozzle and working at high pressure conditions. Then, a supercritical mixture of ethanol and carbon dioxide is used to transport lipids inside a formation vessel to surround the droplets. Inverted micelles are created in the fly; finally, they fall in a water pool set at the bottom of the formation vessel and lipidic double layers of liposomes are created.

Nanometric vesicles (100-300 nm) were obtained, with the possibility to tune their mean diameter changing the operative pressure (100-180 bar), nozzle diameter (40-100 μm) or the Gas to Liquid Ratio on mass base (1-6 GLR). The continuity of the process guaranteed the production of replicable liposome suspensions, that were stable over an observation period of 1 year. Compounds such as proteins (bovine serum albumin), dyes (fluorescein), antibiotics (ampicillin, ofloxacin), essential oils (eugenol), antioxidants (farnesol, linalool, limonene) and dietary supplements (olive pomace extract) were entrapped in the SuperLip produced liposomes, obtaining encapsulation efficiencies up to 99 %. A scale up of SuperLip will be provided in the future, with the aim of the commercialization of industrial volumes of liposome-based products.

Keywords

Liposomes, drug carriers, dense gas methods, process parameter optimization

Abbreviation list

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

LWR: Lipid to Water Ratio

INTRODUCTION

Several drug delivery systems have been studied in the last decades. Among these, liposomes are spherical drug carriers that spontaneously organize in an aqueous inner core surrounded by a lipidic double layer. These vesicles have a diameter that can range between 100 nm to several microns [1]; they are biocompatible with human body for their similarity with cell structure [2]. In details, their double layer can be defined as a membrane; for this reason, liposomes can be described as artificial cells of spherical shape and smooth surface. Liposomes have been employed in numerous applications in pharmaceutical [3], cosmetic [4], and dietary supplement field [5]. In particular, they can be used as Trojan horses [6] to transport hydrophilic and lipophilic compounds directly to the target tissue [7], reducing drug side effects and enhancing drug bioavailability. Despite the recognized importance of these drug carriers, their introduction into the market was not easy. The main reason is linked to the first production mechanism that was developed by the hematologist and biophysicist Dr. Alec Bangham of Babraham Institute in Cambridge in 1965.

Many conventional techniques have been developed for the production of liposomes. But they generally consist in several preparation steps, with batch layout and little reproducibility of the process. Moreover, they are characterized by low encapsulation efficiencies and micrometric dimensions. Supercritical assisted techniques were also proposed to overcome the previous described limitations, trying to take advantage of the high diffusivity of supercritical carbon dioxide. Also, supercritical fluids are clean substitutes of the organic solvents in particle formation processes. But, also the newly proposed processes suffered of many disadvantages such as difficult control of particle size distribution and multi-step production [8].

For this reason, a novel dense gas process has been designed and developed to overcome most part of the limitations linked to the production of liposomes [9]. This novel process was called Supercritical assisted Liposome formation (SuperLip). Considering the limitations of the previous techniques, a novel approach was attempted starting from the mechanism that involved liposomes production. Differently from the other processes, first droplets of water were created through a spray atomization and then they were fast surrounded by phospholipids. Indeed, the liposomes were created around water droplets. In this way, the entrapped compounds stayed confined in the water droplet during the formation of the double lipidic compartment around it. The real key and advantage of this process is that it inverted the traditional production steps of liposomes by first creating the droplet and then the lipid barrier [10].

The aim of this work is to show some interesting results obtained with SuperLip process during the last years of experiments. The idea at the basis of this project was to produce liposomes of nanometric dimensions with encapsulation efficiencies much higher than the ones obtained with

conventional and other dense gas methods. Also, the process was designed and developed in a continuous operative layout to guarantee the reproducibility of vesicles obtained.

Using SuperLip process and taking advantage of the high diffusion coefficient of supercritical carbon dioxide, the production of liposomes was attempted studying the effects of the variation of many operative parameters such as pressure, temperature, lipid concentration, nozzle diameter, water flow rate and Gas to Liquid Ratio. Also, the lipidic composition of the double lipidic layer was also varied and optimized by including cholesterol and compacting the drug carrier structures. Also, the encapsulation of proteins such as bovine serum albumin, dyes as fluorescein [11], antioxidants such as eugenol, lipoic acid [12], limonene, linalool, farnesol, antibiotics such as ampicillin, ofloxacin, vancomycin, theophylline, food dietary supplements such as olive pomace extracts [13] was attempted. A general overview of these experiments and results will be provided throughout this work.

MATERIALS AND METHODS

Materials

L- α -Phosphatidylcholine (PC) from egg yolk (99 % pure) was purchased by Sigma-Aldrich, Milan, Italy. Lipids were stocked in absence of light at the temperature of -4 °C. Phospholipids were dissolved in ethanol obtained by Sigma-Aldrich, Milan, Italy (> 99.8% pure). Carbon dioxide was provided by Morlando Group, Naples, Italy (> 99.4% pure, at gas-liquid equilibrium). For the preparation of liposomes, distilled water was self-produced through a lab-scale distillatory. Hydrophilic (fluorescein, bovine serum albumin, ofloxacin, theophylline, ampicillin, vancomycin), amphoteric (eugenol) and lipophilic compounds (lipoic acid, farnesol, limonene, linalool, cholesterol) were all bought from Sigma Aldrich, Milan, and used as received.

Apparatus

The proposed innovative technique has been designed and developed in lab-scale layout. Carbon dioxide was pumped from a reservoir using a Ecoflow pump, mod. LDC-M-2, Lewa, Germany. Phospholipids were dissolved in ethanol at different volumetric concentrations. Then, the ethanol solution was pumped to the system with a high pressure precision pump, Model 305, Gilson, France. Carbon dioxide and ethanol were mixed together in a thermally heated stainless steel saturator at the pressure of 100 bar and at the temperature of 40 °C.

In those conditions, an expanded liquid was created using Berl saddles loaded in the saturator and working at a fixed Gas to Liquid Ratio. The supercritical mixture transported the lipids inside a stainless steel formation vessel. Another feeding pipe delivered water solution in which a hydrophilic compound has been previously dissolved.

Figure 1 described in details the phenomena occurring in the formation vessel. In (1) the water solution is continuously sprayed inside the vessel through a nozzle of 80 μm . Water droplets were created and the expanded liquid transported the phospholipids inside the vessel (2) and a water bulk was created on the bottom. The lipids surrounded the water droplets and created a first layer around them: inverted micelles were created (3). A second layer of phospholipids was then created around the first one in the fly (4). The solvent was removed from the top of the formation vessel through a decompression step with the use of a stainless steel separator. Liposomes were collected from the bottom of the formation vessel in aqueous bulk.

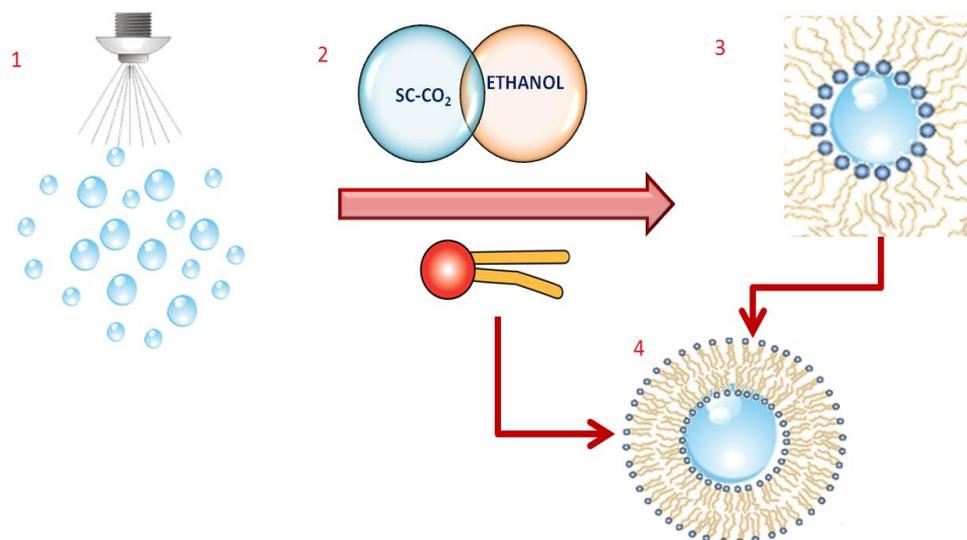


Figure 1. Supercritical assisted Liposome formation: schematic representation of the process mechanism

Methods

Liposomes were characterized with Dynamic Light Scattering (Mod. Zetasizer Nano S, Worcestershire, UK), to measure the mean diameter (MD) and standard deviation (SD).

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 an aluminum stub and left to dry for at least 2 days. Then, the samples were covered with gold through a sputter coated with a thickness of 250 Å, model B7341, Agar Scientific, Stansted, United Kingdom.

The encapsulation efficiency was indirectly measured by the supernatant after the centrifugation of samples, as reported in literature using UV-Vis spectrophotometer. The value of the encapsulation efficiency was then calculated with the following equation:

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

In particular, $ppm_{theoretical}$ is the drug amount dissolved in the water phase at the beginning of the process. instead, $ppm_{supernatant}$ stands for the not entrapped compound. UV-Vis spectrophotometer was also used to perform drug release tests.

RESULTS

SuperLip process was successfully used to entrap compounds of different characteristics and properties, such as antibiotics, dietary supplements, proteins, antioxidants and vitamins. In **Table 1**, the list of all the compounds for which the encapsulation has been attempted is reported. The proposed molecules have been divided into three groups: water core entrapment, lipidic layer encapsulation and encapsulation in both compartments. All the encapsulation tests were performed with a Drug to Lipid Ratio (DLR) of 15 % on mass base. Mean dimensions and average encapsulation efficiencies were also reported in **Table 1**.

Table 1. List of compounds entrapped into liposomes with their Mean Diameter (MD), average Encapsulation Efficiencies (EE) and compartment of encapsulation.

Compound	Liposome Compartment	Mean diameter nm \pm SD	Average Encapsulation Efficiency, %
Fluorescein	Water core	277 \pm 61	90.0
Bovine Serum Albumin		245 \pm 73	93.9
Ofloxacin		256 \pm 71	86.2
Theophylline		136 \pm 86	98.0
Ampicillin		313 \pm 69	99.0
Vancomycin		180 \pm 48	60.0
Olive pomace extract		264 \pm 98	58.1
Eugenol*	Both	196 \pm 76	92.5
Eugenol**		234 \pm 101	86.3
Linalool	Lipidic layer	128 \pm 49	54.7
Lipoic acid		109 \pm 49	62.8
Limonene		159 \pm 44	86.8
Farnesol		126 \pm 35	74.0
Cholesterol		140 \pm 27	96.3
Phosphatidylethanolamine		150 \pm 25	95.1

**Eugenol entrapped in the inner water core of liposomes*

***Eugenol entrapped in the double lipidic layer of liposomes*

As reported in **Table 1**, hydrophilic compounds were entrapped in the double layer of phospholipids with different drug theoretical loadings. In particular, the smallest mean diameter was related to theophylline loaded vesicles (136 \pm 86 nm), while the largest was represented by ampicillin loaded liposomes (313 \pm 69 nm). Liposomes of nanometric dimensions were produced in all experiments, with encapsulation efficiencies high up to 99 % for ampicillin loaded liposomes. Most part of hydrophilic compounds was encapsulated with an EE higher than 90 %. The only two less successful encapsulation tests were performed for vancomycin (60 %) and olive pomace extract (58 %). The amphiphilic chosen compound was eugenol, that was entrapped both in the lipidic layer (86.3 %) and in the inner water core (92.5 %). Water entrapped eugenol caused the production of liposomes with a mean diameter of 196 \pm 76 nm while the lipidic layer entrapped eugenol produced more dispersed samples of liposomes, with a mean diameter of 234 \pm 101 nm. Most part of chosen lipophilic compounds were antioxidants particularly sensible to light and oxygen exposure. The obtained liposomes were substantially smaller, with mean diameters from 109 \pm 49 nm (lipoic acid) to 159 \pm 44 nm (limonene). The samples were less dispersed while average encapsulation efficiencies were smaller than hydrophilic entrapped compounds, from a minimum of 54.7 % (linalool) to 86.8 % (limonene). Cholesterol and phosphatidylethanolamine were also entrapped in the lipidic layer of liposomes, and a more compact structure was obtained, with encapsulation efficiencies of 96.3 % (cholesterol) and 95.1 % (phosphatidylethanolamine).

After these feasibility tests, SuperLip technique was recognized to be versatile for the encapsulation of active principles of different nature. This could open applications in different commercial fields of applications, such as pharmaceutical, cosmetic and nutraceutical.

Characterization

An example of liposomes loaded with ofloxacin (hydrophilic) was observed using Field Emission Scanning Electron Microscope (**Figure 2**).

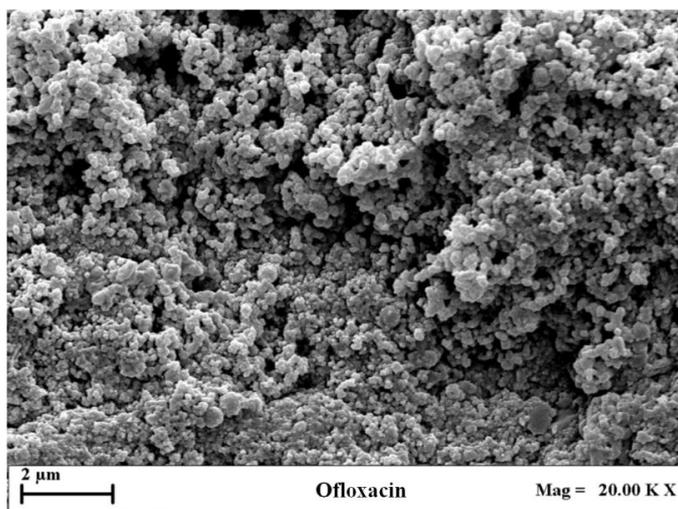


Figure 2. Field Emission-Scanning Electron Microscope of liposomes loaded with ofloxacin

The samples reported in **Figure 2** showed that liposomes are spherical and have a smooth surface. Their nanometric dimensions and size analysis with dynamic light scattering reported in **Table 1** were confirmed. The inclusion of a hydrophilic compound in the inner core of liposomes did not significantly modify liposomes structure and morphology. However, aggregation phenomena occurred; this was probably due to the dehydration and metallization processes that were performed on the samples for microscopic observation.

Drug release tests were performed at 37 °C for ampicillin and ofloxacin loaded vesicles. Release profiles have been compared in **Figure 3**.

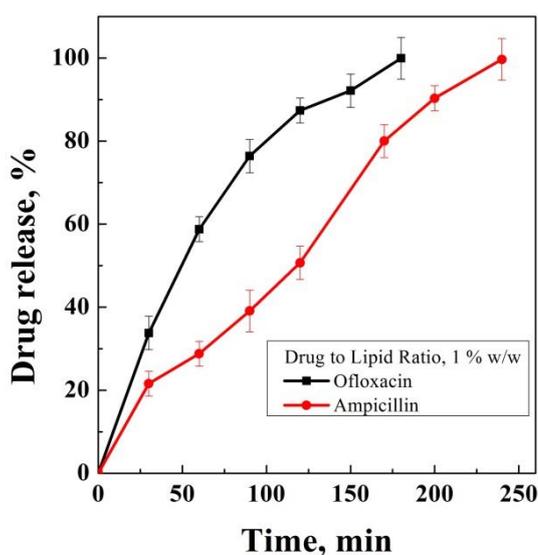


Figure 3 Drug release comparison of ofloxacin (black) and ampicillin (red) loaded liposomes

Ofloxacin and ampicillin loaded vesicles released the entrapped drug in a controlled manner. In particular ofloxacin was completely released in 180 min; whereas, the release of ampicillin was completed after 240 min. These results are the demonstration that antibiotics loaded liposomes are suitable for ocular delivery providing a sustained drug release and improving drug ocular bioavailability.

Eugenol loaded liposomes were also characterized by stability tests. Mean size of vesicles were monitored over the time to measure the stability in water suspension. Data are plotted as a function of time in **Figure 4**.

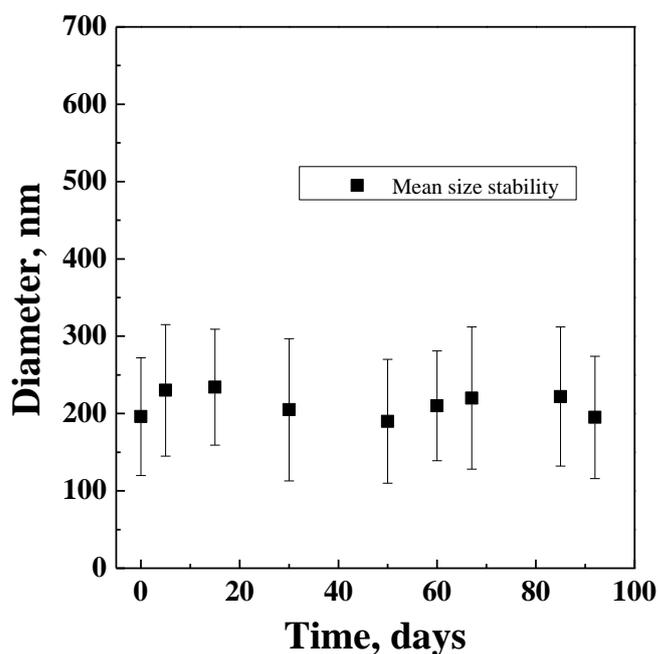


Figure 4 Mean size of vesicles loaded with eugenol

The mean diameter of liposomes was practically constant for more than 3 months of observation when stored at a temperature of 4 °C in the dark. The fluctuations reported in the graph can be ascribed to dynamic phenomena of aggregation and disaggregation of vesicles in the aqueous external bulk.

CONCLUSIONS

Supercritical assisted Liposome formation is a dense gas based techniques that overcame many limitations linked to traditional production methods of liposomes. Exploiting the high diffusion coefficient of carbon dioxide, liposomes of about 100-300 nm were obtained, with a good control of particle size distribution. A huge number of hydrophilic and lipophilic compounds was entrapped in liposomes, confirming that the process is versatile and scalable to industrial level, also thanks to its continuous layout scheme. The fact that liposomes are formed in the fly offered many advantages to the process, such as the high encapsulation efficiency of entrapped compounds.

Future developments will involve the creation of surface-modified liposomes with improved target-delivery functions. Moreover, coated liposomes will be produced to enhance drug protection in long circulating drug delivery systems.

ACKNOWLEDGEMENTS

The authors want to acknowledge the bachelor and master degree students which worked on the production of liposomes with SuperLip process: Daniela Apicella, Annabella Bruno, Marina Iorio, Luca Vitale, Alessandra Saturnino and Paola Russo.

REFERENCES

- [1] SHARMA, D., ALI, A. A. E., TRIVEDI, L. R., An Updated Review on: Liposomes as drug delivery system, *PharmaTutor*, Vol. 6, 2018, p. 50-62.
- [2] MALLICK, S., CHOI, J. S., Liposomes: versatile and biocompatible nanovesicles for efficient biomolecules delivery, *Journal of nanoscience and nanotechnology*, Vol. 14, 2014, p. 755-765.
- [3] TORCHILIN, V. P., Recent advances with liposomes as pharmaceutical carriers, *Nature reviews Drug discovery*, Vol. 4, 2005, p. 145-160.
- [4] MÜLLER, R. H., PETERSEN, R. D., HOMMOSS, A., PARDEIKE, J., Nanostructured lipid carriers (NLC) in cosmetic dermal products, *Advanced drug delivery reviews*, Vol. 59, 2007, p. 522-530.
- [5] KELLER, B. C., Liposomes in nutrition, *Trends in Food Science & Technology*, Vol. 12, 2001, p. 25-31.
- [6] BANGHAM, A. D., Surrogate cells or Trojan horses, The discovery of liposomes, *BioEssays*. Vol. 17, 1995, p. 1081-1088.
- [7] JOSEPH, J., Experimental optimization of Lornoxicam liposomes for sustained topical delivery, *European Journal of Pharmaceutical Sciences*, Vol. 112, 2018, p. 38-51.
- [8] MOZAFARI, M. R., Liposomes: an overview of manufacturing techniques, *Cellular and Molecular Biology Letters*, Vol. 10, 2005, p. 711-719.
- [10] SANTO, I. E., CAMPARDELLI, R., ALBUQUERQUE, E. C., DE MELO, S. V., DELLA PORTA, G., REVERCHON, E., Liposomes preparation using a supercritical fluid assisted continuous process, *Chemical Engineering Journal*, Vol. 249, 2014, p. 153-159.
- [11] CAMPARDELLI, R., SANTO, I. E., ALBUQUERQUE, E. C., DE MELO, S. V., DELLA PORTA, G., REVERCHON, E., Efficient encapsulation of proteins in submicro liposomes using a supercritical fluid assisted continuous process, *The Journal of Supercritical Fluids*, Vol. 107, 2016, p. 163-169.
- [12] CAMPARDELLI, R., TRUCILLO, P., REVERCHON, E., A supercritical fluid-based process for the production of fluorescein-loaded liposomes, *Industrial & Engineering Chemistry Research*, Vol. 55, 2016, p. 5359-5365.
- [13] TRUCILLO, P., CAMPARDELLI, R., REVERCHON, E., Production of liposomes loaded with antioxidants using a supercritical CO₂ assisted process, *Powder Technology*, Vol. 323, 2018, p. 155-162.
- [14] TRUCILLO, P., CAMPARDELLI, R., ALIAKBARIAN, B., PEREGO, P., REVERCHON, E., Supercritical assisted process for the encapsulation of olive pomace extract into liposomes, *The Journal of Supercritical Fluids*, Vol. 135, 2018, p. 152-159.