Antioxidant Loaded Nanoemulsions Entrapped In Liposomes Produced With A Supercritical Assisted Technique

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ABSTRACT

Antioxidant compounds such as linalool and limonene are nowadays largely employed in the nutraceutical and cosmetic field to preserve human health. Although they are characterized by a remarkably strong antioxidant activity, they are particularly sensible to oxidation and poorly absorbed after administration. One possible way to improve their bioavailability is to encapsulate them into drug carriers. Among the great variety of entrapping vehicles, liposomes are commonly used for their biocompatibility with human cell barriers. These vesicles are characterized by a inner water core surrounded by a double layer of phospholipids. Liposomes are also versatile objects; in fact, they have been employed to deliver proteins, enzymes, antioxidants, flavors and vitamins.

To overcome the common limitations linked to traditional liposomes production methods, a supercritical assisted process called SuperLip has been developed for the production of liposomes of nanometric dimensions, with a good control of particle size distribution, high encapsulation efficiencies, low solvent residue through a continuous, replicable and cheap technique.

In this work, lipophilic antioxidants were entrapped in liposome barriers using SuperLip, with encapsulation efficiencies up to 50 %. In fact, the contact with external solvents caused a faster denaturation of the molecules than inner core encapsulation. Another possibility studied in this work was to dissolve lipophilic compounds into an oil phase of an Oil in Water (O/W) emulsion. Operating in this manner, encapsulation efficiencies up to 99 % were obtained for linalool and limonene. Monodispersed liposomes with mean dimensions of 400 nm, stable over months of observation, were produced.

Keywords

Liposomes, emulsions, antioxidant compounds

Abbreviation list

PC: L-α-Phosphatidylcholine, **EE**: Encapsulation Efficiency, **EL**: Expanded Liquid

MD: Mean Dimensions, **SD**: Standard Deviation, **PDI**: Polydispersion Index,

O/W: Oil in Water (emulsion)

INTRODUCTION

Liposomes are spherical drug delivery systems composed by a double lipidic layer of phospholipids that surrounds an inner volume of water. Liposomes attracted scientific and industrial attention for their biocompatibility [1], biodegradability [2] and non-invasive approach [3] with human cells and their versatile applications in food [4], cosmetic [5] and pharmaceutical [6] application field, with a great industrial impact for commercialization. Lipidic vesicles are nowadays the most studied nanocarriers for their potentiality to entrap hydrophilic (aqueous core) and lipophilic (lipidic compartment) molecules, enhancing drug bioavailability, protecting them from degradation and improving therapeutic efficacy of entrapped drugs [7].

Many vesicles preparation methods have been proposed in literature in the last decades [8], but there are many related drawbacks such as low encapsulation efficiency, batch layout, low replicability and less absorbable micrometric vesicle formation. Some newly production methods tried to overcome these problems through mechanical dispersion [9] with sonication. However, even if it managed to obtain single unilamellar liposomes (100-200 nm), this method caused disruption of vesicles with loss of internal drug content. Other methods used the solvent dispersion to enhance the mixture of lipids into the aqueous phase for fast liposome production; but, in this case, high amounts of solvent residue were a problem.

A process called Supercritical assisted Liposome formation (SuperLip) has been developed to try to overcome the problems that were still pending on liposomes production. With this process, nanometric liposomes were obtained with a good control of particle size distribution and entrapment efficiencies up to 99 % were obtained for hydrophilic compounds. SuperLip was used for the successful encapsulation of many molecules for different fields of application: proteins such as Bovine Serum Albumin [10], dyes such as fluorescein [11], antibiotics such as ampicillin, ofloxacin and theophylline [12], food supplements such as olive pomace extract, essential oils and amphiphilic and lipophilic antioxidants such as eugenol and lipoic acid.

The antioxidant selected for this work are lipophilic. In a previous study, it was demonstrated that the inhibition power of antioxidant compounds is better preserved if the molecules are entrapped in the inner aqueous core of liposomes [13].

The aim of this work is to exploit antioxidant compounds such as limonene and linalool, entrapping them into liposomes using SuperLip technique. Linalool is a terpene alcohol that is found in nature in anise, pepper and fennel plants. It is characterized not only by antioxidant power, but also by antitumor, antidepression and antimicrobial activities [14]. Limonene is another very common terpene because it is the main component of several citrus oils. Is it a widely used flavoring agent for fruit juices, ice creams and soft drinks; it also has anti-carcinogenic effects [15].

In this paper we present a new approach for the encapsulation of lipidic compounds, directly in the inner liposomes aqueous core. Antioxidants were dissolved into the oil phase of an Oil in Water (O/W) emulsion. Then, the emulsion was entrapped into the inner hydrophilic core of liposomes produced with SuperLip. In this way, the antioxidant properties would be better preserved. Another objective of this work is to provide a comparison of these experiments with the encapsulation into the lipidic layer. Finally, a simultaneous encapsulation of limonene and linalool into emulsion loaded liposomes has been planned.

MATERIALS AND METHODS

Materials

L-α-Phosphatidylcholine from egg yolk (PC) was supplied by Sigma-Aldrich, Italy (99% pure, lyophilized powder). Ethanol used to dissolve phospholipids was purchased from Sigma-Aldrich (≥99.8%). Carbon dioxide (CO_2) was provided by Morlando Group, Italy (>99.4% pure). Distilled water, used in all formulations was self-produced through a distillatory in our laboratories. Isopropyl myristate (<90% pure), surfactant Tween 80 and two antioxidants, limonene and linalool, were provided by Sigma-Aldrich, Italy. All the materials were used as received.

Apparatus

The supercritical technology SuperLip was developed according to a lab-scale configuration. In details, two piping lines fed pure carbon dioxide (through Ecoflow pump, mod. LDC-M-2, Lewa, Germany) and an ethanolic solution (through high pressure precision pump, Model 305, Gilson, France) in which phospholipids and lipophilic compound were dissolved. This two feeding lines were mixed in a saturator (loaded with stainless steel Berl saddles and thermally heated) with a Gas to Liquid Ratio fixed to 2.4. Once the Expanded Liquid (EL) was formed, it was fed to a high pressure formation vessel. A third feeding line delivered an aqueous solution or the emulsion in which the antioxidant compound is contained. The emulsion was continuously sprayed with a nozzle of 80 μm diameter. In this manner, droplets were created and rapidly surrounded by phospholipids during precipitation. A water bulk was created on the bottom of the formation vessel to collect the vesicles created in the fly. The organic solvent was removed from the top of the formation vessel through a decompression step; in this way, carbon dioxide was separated from ethanol. Carbon dioxide flow rate was measured using a rotameter (mod. N.5-2500, Serval 115022, ASA, Italy). Liposomes were collected from the bottom of the formation vessel in an aqueous bulk.

Emulsion preparation

Water in Oil (W/O) emulsions were prepared following this standard procedure: water phase was obtained dissolving 0.18 g of surfactant Tween 80 in 90 mL distilled water and the solution was stirred with a magnetic stirrer at 250 rpm for 30 min at room temperature. The Oil phase was prepared by dissolving 5, 10 and 15 % of the chosen antioxidants (on lipid mass base) in 10 g isopropyl myristate and the solution was agitated at the same conditions. Then, the Oil phase was kindly added to the Water phase and agitated using an emulsifier (mod. L4RT, Silverstone, USA), working at 7000 rpm for 6 min. The obtained emulsion was finally fed to SuperLip apparatus.

Methods

Liposome suspensions were characterized using Dynamic Light Scattering (Mod. Zetasizer Nano S, Worcestershire, U.K.), to measure Mean Dimensions (MD), Polydispersion Index (PDI), Standard Deviation (SD) and zeta potential to analyze the average surface charge of lipidic vesicles.

Liposome morphology was observed with a Field Emission-Scanning Electron Microscope (FE-SEM, model LEO 1525, Carl Zeiss SMT AG, Oberkochen, Germany). Samples were prepared by spreading a drop of liposome suspension on an aluminum stub and leaving it to dry in air for 2-3 days. Dry liposome suspension was then covered with gold, using a sputter coater (thickness of 250 Å, model B7341, Agar Scientific, Stansted, U.K.).
Not entrapped linalool was detected from the supernatant after centrifugation [16] at the wavelength of 233 nm [17] using UV-Vis spectrophotometer and the encapsulation efficiency was calculated as follows:

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

in which $ppm_{theoretical}$ is the amount of antioxidant dissolved in the initial phase and $ppm_{supernatant}$ represents the not entrapped compound. Instead, limonene was detected at the wavelength of 270 mn. The absorption peaks were detected after scanning a 100 ppm solution in a range among 200 and 800 nm. The measurements were performed in triplicates. Data were shown as mean value ± standard deviation.

RESULTS

In this work SuperLip process was employed to produce liposomes loaded with antioxidant compounds. The compounds chosen for this study were limonene and linalool, which are two terpenes particularly sensible to oxygen, light and heat exposure. These lipophilic compounds have been previously entrapped in the double lipidic layer using the same process, but the Encapsulation Efficiencies (EE) were lower than 50 %. Moreover, they are particularly soluble in supercritical carbon dioxide and for this reason they can be extracted, during the process, reducing the average EE.

To increase the encapsulation efficiency of these antioxidants into liposomes, the lipophilic drugs were dissolved in isopropyl myristate and the obtained solution was emulsified in water. The Oil in Water (O/W) emulsion was then entrapped into liposomes using SuperLip technology. In the first set of experiments, liposomes were produced with an increasing theoretical loading of limonene. Then, the same drug concentrations were used to produce liposomes loaded with linalool. Finally, a simultaneous encapsulation of limonene and linalool was attempted with a fixed theoretical drug concentration (10 % w/w on lipid mass base).

SuperLip operative parameters were fixed as follows: pressure to 100 bar, temperature 40 °C, CO_2 flow rate 6.5 g/min, water flow rate 10 mL/min, ethanol flow rate 3.5 mL/min, lipidic concentration of 5 mg/mL in the ethanol solution. In **Table 1**, Mean Diameters (MD) and Encapsulation Efficiencies (EE) of limonene and linalool were reported.

Table 1. Theoretical loadings, Mean Diameters (MD) and Encapsulation Efficiencies (EE) of SuperLip experiments for limonene, linalool and limonene+linalool loading into liposomes

Compound	Theoretical loading [%]	Mean diameter [nm ± SD]	Encapsulation Efficiency [%]
Limonene	5	655 ± 507	60
	10	492 ± 251	90
	15	397 ± 120	92
Linalool	5	424 ± 141	96

	10	521 ± 188	97	
	15	605 ± 235	99	
Limonene + Linalool	10	489 ± 59	99	99

The Water in Oil (W/O) emulsions were stable before and after liposomes entrapment. From results reported in **Table 1**, it was possible to observe that liposome mean diameters decreased with the increase of limonene theoretical loading, from 655 ± 507 nm of the 5% loaded sample to the 397 ± 120 nm for the sample with the highest drug concentration. Encapsulation efficiencies of entrapped compounds were higher than liposomes produced with conventional methods (< 40 %). Moreover, they increased with the increase of theoretical loading, from a minimum of 60 % for liposomes loaded with 5 % of limonene up to a maximum of 92 % for vesicles loaded with 15 % of limonene.

Differently from the first set of experiments, linalool loaded liposomes showed a mean diameter that increased with the theoretical loading of linalool, from a minimum of 424 ± 141 nm to a maximum of 605 ± 235 nm. This different behavior probably depends on the molecular interaction among lipids and antioxidant entrapped compounds. Instead, the trend related to the encapsulation efficiencies of linalool loaded liposomes was still increasing from a minimum of 96 % to a maximum of 99 %, as reported for limonene vesicles.

A final effect of simultaneous encapsulation of linalool and limonene on mean diameters has also been studied and particle size distribution of produced liposomes was reported in **Figure 1**.

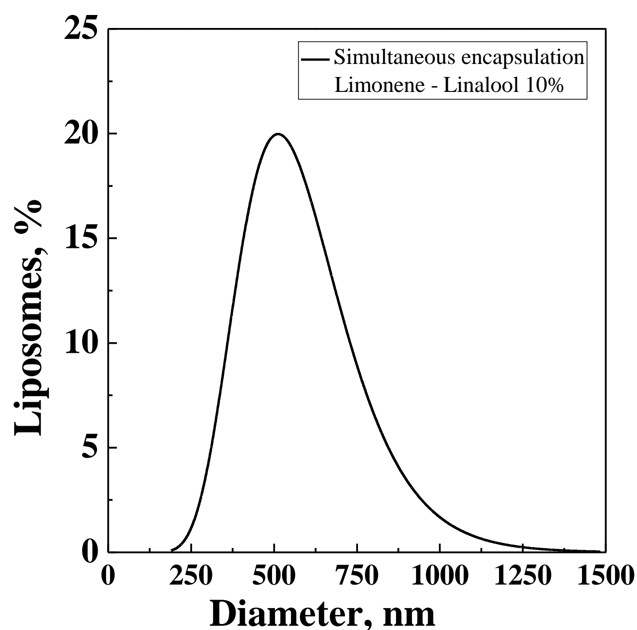

Figure 1. Particle Size Distribution of liposomes simultaneously loaded with 10 % Limonene and 10 % Linalool on lipid mass base

As reported in **Figure 1** and **Table 1**, liposomes loaded with 10 % limonene and 10 % linalool on lipid mass base were 489 ± 59 nm large. In this case, the simultaneous presence of the two antioxidants reduced significantly the dispersion of the particle size distribution. This was probably due to the sum of the effects of limonene and linalool during simultaneous encapsulation. The encapsulation efficiency was definitely optimized since it was 99 % both for limonene and linalool.

This confirmed that the encapsulation of drugs is favored in the case of a mixture of the two compounds.

Liposomes produced were observed using Field Emission-Scanning Electron Microscope (FE-SEM) to observe the effect of SuperLip process on the morphology of the vesicles. In **Figure 2**, a FE-SEM image of liposome suspension loaded with 10 % limonene was reported.

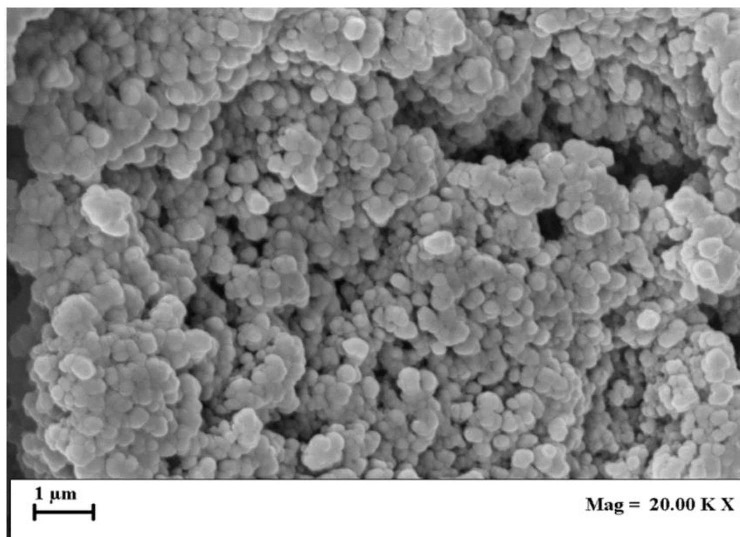

Figure 2. Field Emission-Scanning Electron Microscope of liposomes loaded with 10 % limonene on lipid mass base

Figure 1 confirmed mean dimensions reported in **Table 1** for liposomes suspensions loaded with 5 % limonene. Mean dimensions are sub-micrometric, the surface is smooth and the shape is spherical. Vesicles size distribution is homogeneous. The elevate polydispersion of sample loaded with 5 % limonene is probably due to the interaction between the antioxidant and lipids, with a general tendency with aggregation phenomena. The following FE-SEM observation was performed on 10 % linalool loaded samples (**Figure 3**).

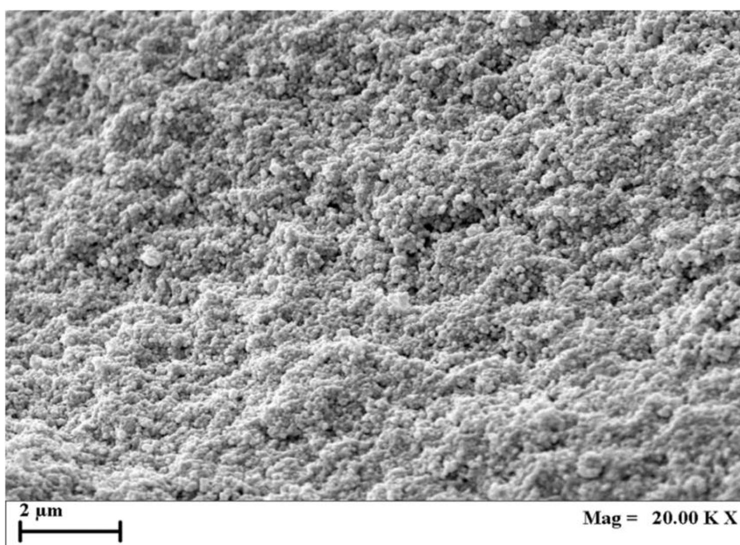

Figure 3. Field Emission-Scanning Electron Microscope of liposomes loaded with 10 % linalool on lipid mass base

Mean dimensions of liposomes loaded with linalool still confirmed the mean dimensions reported in **Table 1**. In particular, the morphology of vesicles did not show any significant variation after the

antioxidant encapsulation. As a general comment, liposomes produced for this study showed an almost evident vesicles aggregation phenomenon. The reason is probably linked to the presence of isopropyl myristate used to prepare emulsions entrapped in the inner aqueous core of liposomes.

CONCLUSIONS

In this study, SuperLip process was employed to encapsulate limonene and linalool into liposomes. Since these two compounds are lipophilic, they were dissolved into isopropyl myristate and emulsified in pure water. This O/W emulsion was entrapped into the aqueous core of liposomes. The two sets of experiments demonstrated that it was possible to entrap O/W emulsions into liposomes without damaging the antioxidant compound dissolved in the inner phase. In all the experiments, liposomes of sub-micrometric dimensions were obtained, with an encapsulation efficiency increased up to 99 % by increasing the theoretical loading of the compound. The encapsulation efficiency (EE) was also higher than liposomes produced with conventional methods. Finally, the simultaneous entrapment of the two antioxidants was the most successful experiment of this study because it reached the EE of 99 % for both compounds.

Future prospective will be characterized by stability studies by measuring the mean diameter of liposomes over a time of 6 months. Moreover, drug release will be performed to study the pharmacokinetics from emulsions. The inhibition of the antioxidant power of these compounds will be measured either in the case of inner core entrapment, either for the encapsulation in the double lipidic compartment.

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