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Production of liposomes loaded with antioxidants using a supercritical CO_2 assisted process

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ABSTRACT

To preserve benefic effects of antioxidants, they can be protected using a drug carrier. Conventional processes for the production of antioxidants-loaded carriers suffer of some drawbacks that can be overcome using Supercritical Assisted Liposome Formation (SuperLip). In this work, SuperLip was used to encapsulate an amphiphilic (eugenol, EUG) and a lipophilic (α-lipoic acid, ALA) antioxidant in liposome vesicles. EUG loaded liposomes were produced with a mean diameter of about 200 nm and demonstrated to be stable for at least 40 days. Eugenol was entrapped in the inner core and in the lipophilic double layer, with Encapsulation Efficiencies (EE) up to 94.2 ± 2.9%. Liposomes with a mean diameter of about 230 nm loaded with ALA in the lipidic layer were also successfully produced, with EE up to 68.1 ± 6.1%.

Free-radical scavenging assay results indicated that EUG and ALA antioxidant power was preserved after processing; a reduction of the inhibition power, compared to the un-processed molecule, in the range between 6% and 13% was obtained. Drug release tests were also performed at 30 °C and 60 °C, showing that these liposomes are temperature sensitive.

1. Introduction

The controlled delivery of antioxidants [1] is of interest for therapeutic [2] and cosmetic [3] applications. For example, eugenol (EUG, 4-allyl-2-methoxyphenol) has a remarkable antioxidant activity and antibacterial and antifungal properties [4]. It is generally used as food flavoring agent, as additive to fragrances and in active packaging applications [5–7]. α-Lipoic acid (ALA) [8] also possesses an antioxidant activity and it is often used in human diet for its functional properties [9]. Moreover, it can be used as a therapeutic agent to prevent some pathologies, like diabetes [10,11]. However, antioxidants are generally unstable and sensitive to oxygen, light and heat [12-15]. For these reasons, it is preferable to protect and deliver them using a carrier.

It is well known that nanoencapsulation offers various benefits such as enhanced stability, protection against oxidation, retention of volatile compounds, reduced toxic effects and enhanced bioavailability [16,17]. Liposomes are lipidic vesicles characterized by a hydrophilic inner water core surrounded by a lipophilic double layer of phospholipids and can be used as efficient encapsulation vehicles [18]. These lipid carriers can mimic the biophysical properties of living cells [19] and can be used to vehiculate proteins, dyes, anti-cancer, DNA and antioxidant compounds to target tissues [20,21].

EUG loaded liposomes were produced using thin film hydration and reversed-phase evaporation methods [22,23]. However, these techniques suffer of many drawbacks because they require complex post-treatments steps. The produced liposomes were not homogeneous and Particle Size Distribution (PSD) was difficult to replicate. Some authors attempted to protect EUG using the encapsulation in nanoparticles, nanocapsules, microcapsules and liposomes [24]. Encapsulation Efficiencies (EE) up to 86.6% were reported for eugenol encapsulation into liposomes [23]. However, the preservation of the antioxidant activity was not verified. Some authors tried to entrap ALA in microspheres, using chitosan as the polymeric matrix [25], obtaining micrometric ALA-loaded particles with 55% encapsulation efficiency; however, a reduction of antioxidant activity of lipoic acid of 25% after encapsulation in the chitosan matrix was observed.

Supercritical CO_2 (SC-CO_2) has been successfully used in some non-traditional processes such as extraction [26,27], micronization [28], desorption [29,30] and membranes formation [31] taking advantage of SC-CO_2 high diffusion coefficients, low viscosity and near zero surface tension. These properties favor a fast processing and a good control of material properties [32].

Some supercritical based processes have also been developed for the production of liposomes [33–35]; but, they were not particularly successful, especially from the point of view of the encapsulation efficiency. The recently proposed SuperLip process (Supercritical Assisted Liposome Formation) modifies the concepts at the basis of liposomes formation [36]; indeed, during this process, first phospholipids are

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dissolved in ethanol and supercritical CO_2 to form an expanded liquid, then a water solution is atomized in the formation vessel to produce droplets. These droplets are rapidly covered by the phospholipids contained in the fluid phase and liposomes are formed. The encapsulation of hydrophilic compounds with high entrapment efficiency was demonstrated; for example, fluorescein loaded liposomes were produced [37], obtaining Single Unilamellar Liposomes (SUVs) down to 100 nm with EE up to 99%. Theophylline loaded liposomes [38] were also produced, with an encapsulation efficiency of 98%.

Therefore, the aim of this study is to apply SuperLip process to encapsulation of antioxidants to improve their encapsulation efficiency, preserving the antioxidant power of the compounds processed. Two model antioxidants will be tested: EUG and ALA. Until now, SuperLip has not been tested for the entrapment of lipophilic compounds that in principle can only be included in the lipid bilayer; this operation can destabilize the vesicles integrity. Therefore, a further challenge is to validate SuperLip for the incorporation of lipophilic compounds in the lipidic double layer of submicro liposomes. Encapsulation efficiency, free radical scavenging activity and drug release tests will be systematically performed to measure the performance of the process.

2. Materials

Eugenol (MW 164.20 g/mol, purity > 99%, Log $P_{oct/w}$ at 2.13), α-lipoic acid (MW 203.66 g/mol, purity > 99%, powder, Log $P_{oct/w}$ 2.53), ethanol (purity > 99.8%), L-α-phosphatidylcholine from egg yolk (PC, ~60% purity, lyophilized powder), acetic acid (purity > 99%), 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma Aldrich, Milan, Italy. Carbon dioxide was provided by Morlando Group, Naples, Italy (>99.4% purity). Distilled water was produced in our laboratories.

3. Apparatus

Lipids and lipophilic compounds were dissolved in an ethanol solution at a concentration of 5 mg/mL, then, SC-CO_2 and the ethanol solution were fed separately to a heated saturator, where they were mixed to form an expanded liquid (EL) [39]. The expanded liquid was fed to a high pressure stainless steel Formation Vessel (FV) operating at 100 bar and 35 °C or 40 °C. A third feeding line was used to deliver a water solution, containing the hydrophilic drug, inside the formation vessel. The aqueous solution was atomized to generate small droplets, using a 80 μm nozzle. Carbon dioxide was pumped using an Ecoflow pump (mod. LDC-M-2, Lewa, Germany). Water and ethanol solutions were pumped using two high pressure precision pumps (Model 305, Gilson, France). Ethanol solution flow rate was set to 3.5 mL/min and carbon dioxide flow rate was 6.5 g/min, obtaining a Gas to Liquid Ratio (GLR) of 2.4, on mass basis. Water flow rate was 10 mL/min for all the experiments.

Liposome suspension was continuously recovered at the bottom of the formation vessel using an on off valve (Swagelok, model SS43S4, Italy). A decompression step, performed using a back pressure valve (Tescom model 26-1723-44, Italy) separated CO_2 and ethanol downstream the formation vessel, in a stainless-steel separator, operated at 10 bar and 25 °C. A schematic representation of SuperLip is provided as Supplementary document and a detailed description was reported in previous works [36,40].

Tests were performed in triplicates for each experiment.

4. Methods

4.1. Liposomes dimensions

Liposome suspensions were characterized using a 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°. For each liposome sample, 3 measurements were performed reporting their mean values.

4.2. Liposomes morphology

Liposomes morphology and size were also analyzed using a Field Emission-Scanning Electron Microscope (FE-SEM mod. LEO 1525; Carl Zeiss SMT AG, Oberkochen, Germany). Samples were centrifuged at 13000 rpm for 50 min at −4 °C; then, the supernatant was eliminated and the vesicles were re-suspended in distilled water. This operation was repeated twice. Then, a drop of liposome suspension was spread over an adhesive carbon tab placed on an aluminum stub and dried at air for 2 days. Then, the dried samples were coated with a gold layer using a sputter coater (thickness 250 Å, model B7341; Agar Scientific, Stansted, UK), before FE-SEM observation.

Transmission electron microscopy, TEM, (JEOL 1400, 100 kV accelerating voltage) was used with negative staining to investigate the morphology and size of the liposomes produced. A droplet of liposome suspension was placed on a copper grid allowed to sit for 60 s. The droplet was, then, dried with filter paper. A droplet of staining agent was subsequently placed on the top of the grid and left reacting for 30 s, the excess was then removed using filter paper.

4.3. Encapsulation Efficiency measurement

Encapsulation Efficiency (EE) of liposomes was measured using supernatant method [41]. Analysis was performed in triplicates and the resulting values were the average EE. To obtain an accurate measurement of the entrapped drug, liposomes suspensions were ultracentrifuged at 13000 rpm for 50 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 280 nm [42,43] for eugenol and at 325 nm [44,45] for α-lipoic acid. The Encapsulation Efficiency was calculated as the complement to 100 of the percentage of drug present in the supernatant, expressed as in Eq. (1).

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

EE measurements were also performed dissolving directly liposomes. Liposomes suspensions were ultra-centrifuged; supernatant was eliminated and the pellet of concentrated liposomes was re-suspended in 3 mL acetic acid that dissolved the lipid barrier. Then, the content of entrapped compounds was measured using a Micro-volume UV–Vis spectrophotometer (Biospec-nano, Shimadzu Scientific Instruments, Columbia, USA). The two techniques used to measure the EE produced similar results. These two different methods used for the determination of eugenol encapsulation efficiency into produced liposomes allowed to obtain complementary data about eugenol encapsulation; indeed, the supernatant method provided the information about the amount of eugenol not entrapped into liposome; whereas, the pellet method gave the information about the eugenol effectively present inside vesicle inner core. The sum of the two values is 100%. The results obtained using the supernatant methods were confirmed by the results obtained using the pellet method. The encapsulation efficiency values reported in the Results and discussion section are the ones obtained using to the supernatant method.

4.4. Antioxidant inhibition measurement

Antioxidant activity was measured with DPPH method [46]. The aim was to evaluate the hydrogen donation and the radical scavenging