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Production of Solid Lipid Nanoparticles with a Supercritical Fluid Assisted Process

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ABSTRACT
Solid lipid nanoparticles (SLNs) have gained increasing interest as new colloidal system for the delivery of active principles.
In this work, a recently proposed supercritical fluid continuous process, the Supercritical Assisted Injection in a Liquid Antisolvent (SAILA), was proposed for the production of SLNs. The basic principle of this process is the solubilization of a fixed amount of carbon dioxide in a liquid solution containing the solute; then, under determined conditions of pressure and temperature, an expanded liquid solution is formed. The expanded liquid is then atomized directly in a water solution, containing a surfactant, which has the role of the antisolvent. Particles are formed as a consequence of a rapid supersaturation of solutes for the antisolvent effect.
SLNs of soy lecithin, cholesterol and stearic acid were in this work. Acetone and ethanol have been alternatively used as solvents, and Tween 80 + water solution as the antisolvent solution. The effect of lipid concentration in the expanded liquid was studied on particles size distribution and morphology. SLNs with mean dimensions included between 158 ± 53 nm and 326 ± 169 nm were obtained for soy lecithin particles, whereas they were included between 151 ± 74 nm and 207 ± 57 nm for cholesterol and from 364 ± 77 nm to 462 ± 88 nm for stearic acid particles. All the suspensions were stable over 30 days of observation.

Keywords
Solid lipid nanoparticles, supercritical process, pharmaceutical applications

Abbreviations list
SLN: Solid Lipid Nanoparticles, **SCF**: SuperCritical Fluids, **SC-CO_2**: SuperCritical Carbon dioxide, **DLS**: Dynamic Light Scattering, **PSD**: Particle Size Distribution, **EE**: Encapsulation Efficiency, **MD**: Mead Diameter, **SD**: Standard Deviation, **VLE**: Vapor Liquid Equilibrium, **GLR-EL**: Gas to Liquid Ratio of the Expanded Liquid, **SAILA**: Supercritical Assisted Injection in a Liquid Antisolvent, **NP**: NanoParticles, **SLN**: Solid Lipid Nanoparticles

1. Introduction

Research in nanotechnologies has become a rapidly growing field [1]; indeed, in the last few years, the interest in biomedical and pharmaceutical applications has increased exponentially [2-5]. The advancement in these areas led to the emergence of a new multidisciplinary field called nanomedicine [6-9]. Conventional therapies such as surgery and chemotherapy are based on the principle of eliminating tumoral cells; whereas, nanomedicine aims to work more precisely by destroying specific cells or repairing them one by one, avoiding side effects of traditional drug administration [10].

It is well known that development of new drug formulations is not sufficient to ensure progress of pharmaceutical therapies [11]. One of the most challenging issue of drug delivery is to administer the drug directly to the target tissue. Targeted drug delivery ensures greater concentration of the active substance in the required area, providing alternative strategies for more specific therapies. The main peculiarities of Nanoparticles (NP) can be exploited to obtain a passive target delivery; indeed, NPs have little tendency to precipitate into aqueous solutions, they have a greater solubility and have a stronger adhesion to biological surfaces, thus guaranteeing enhanced bioavailability [12, 13].

Solid Lipid Nanoparticles (SLNs) are colloidal systems composed of lipids which are solid at room temperature; in this family of compounds triglycerides, partial glycerides, fatty acids, steroids and waxes are included [14]. SLNs were introduced in 1991 as an alternative to traditional drug transport systems [15-17]. They are very similar to lipid nanoemulsions [18-20] or liposomes [21-23], since they are both suspensions of lipid nanoparticles dispersed in an aqueous surfactant solution; whereas, the main difference among SLNs and lipid nanoemulsions or liposomes is the aggregation status of lipids.

The use of solid lipids for the production of nanocarrier is an extremely interesting approach to obtain drug controlled release, since the mobility of a molecule in a solid lipid is considerably lower than in a liquid oil [24]. Indeed, SLNs are recently used as drug carriers to obtain drug prolonged release; in this way, after administration, a constant concentration of the drug can be maintained in the blood stream. Drug controlled release allows a possible reduction of side effects and reduces the frequency of administrations [25, 26]. Compared to other drug carriers already proposed in literature [27], SLNs present several advantages such as the possibility to entrap hydrophilic and lipophilic compounds; moreover, they do not show problems of toxicity since they are biocompatible with lipids of cell membranes [28].

Currently, different techniques have been proposed for the production of SLNs. Solvent emulsification/evaporation, high-pressure homogenization, and hot/cold homogenization have been the most adopted until now [29, 30]. However, these traditional methods are multi-step and generally involve high temperatures and shear rates, and several cycles at high pressure to reach the nanoscale level. These extreme process conditions lead to an increase of the size and an enlargement of of particle distribution due to particles aggregation problem; furthermore, drug degradation is also possible as a consequence of thermal effects. Other problems of conventional production methods are related to the presence of organic solvent residues that compromises their safety for human use and high kinetic energy content of the obtained particles that promotes their coalescence [31-33].

Supercritical Fluids (SCFs) based techniques have been proposed as an alternative to conventional processes, thanks to their specific characteristics such as solvent power and liquid-like densities, together with gas-like transport properties that can be tuned varying pressure and temperature . They have been successfully applied in several fields such as the micronization [34, 35], extraction

of natural matter [36, 37], impregnation of metals or drugs in aerogels [38, 39], membranes and scaffolds production [40-44]. Supercritical Assisted Injection in a Liquid Antisolvent (SAILA) is an efficient process based on the continuous injection of a solution (formed by an organic solvent and a solid solute) containing controlled quantities of SC-CO_2, in an antisolvent solution. To obtain particles precipitation, the solute has to be soluble in the solvent, but not in the antisolvent; meanwhile, the solvent and the antisolvent have to be completely miscible. SC-CO_2 acts as a co-solute being miscible with the solution solvent-solute, decreasing the mixture viscosity and surface tension that will favor the atomization in the antisolvent. A saturator that contains high surface packings and ensures long residence times is used and a near-equilibrium solution between solute, solvent and CO_2 is formed. Then, this expanded liquid solution is sent to a thin wall injector and sprayed into the precipitation vessel containing the antisolvent (generally water). The mixing of the two fluids produces a rapid supersaturation and particles precipitation, consequently. This process offers several advantages: no thermal degradation (mild temperature are characteristic of supercritical conditions), use of non toxic solvents (water miscible solvents such as acetone and ethanol are generally used), directly producing a stabilized water suspension. Moreover, SAILA provides a good control on particle size distribution with the direct production nanoparticles under the appropriate process conditions. These characteristics already allowed the successful micronization of several compounds [45, 46].
In this work, SAILA technique will be applied for the production of SLNs. Different lipids will be tested, such as soy lecithin, cholesterol and stearic acid. Acetone or ethanol will be used as liquid solvents, whereas water or water + Tween80 surfactant will be employed as anti-solvent. The produced suspensions will be characterized through particle size distributions and scanning electron microscope. The effect of the concentration of lipids dissolved in the starting solution will be studied on dimensions and morphology of nanoparticles produced. Water stability of SLNs suspensions will be observed over a period of 30 days.

2. Materials, apparatus and methods

2.1 Materials

Carbon dioxide (99.9%) was provided by Morlando Group, Naples, Italy. Polysorbate (Tween 80, CAS number 9005-65-6), Sorbitan monolaurate (Span 20, CAS number 1338-39-2), Acetone (AC, purity 99.9%), ethanol (ethanol, purity 98%), soy lecithin (CAS number 8002-43-45), cholesterol (CAS number 57-88-5) and stearic acid (CAS number 57-11-4) were provided by Sigma Aldrich Chemical Co., Italy. Soy lecithin is highly soluble in ethanol (up to 50 mg/mL); whereas, cholesterol and stearic acid are not largely soluble in it. For this reason, they were dissolved in acetone, used as organic solvent in SAILA process substituting ethanol.
All the reagents were used as received. Distilled water was self-provided using a lab distillator and used throughout all the formulations.

2.2 Apparatus

A schematic representation of the SAILA process layout is reported in **Figure 1**. It mainly consists of two feed lines, used to deliver the compressed CO_2 (A) and the liquid solvent (B) to a mixing vessel (saturator, C). Carbon dioxide is cooled, pumped, preheated and delivered to the saturator (C). The liquid mixture formed by the solvent (in which the solid lipid and also a lipidic surfactant, Span 20, can is dissolved), is delivered to the saturator by a membrane pump and is preheated

before the inlet to saturator. The saturator is a high-pressure vessel with an internal volume of 0.15 dm^3, heated by thin band heaters (E) and packed with stainless steel perforated saddles with a high specific surface area (D). It provides a large contacting surface and an adequate residence time (3-5 min, depending on the flow rates) for the liquid-gas mixing. Therefore, an efficient, continuous solubilisation of SC-CO_2 (SuperCritical Carbon dioxide) in the liquid solution is obtained. As a result, an expanded liquid mixture is formed, whose position in the Vapor-Liquid Equilibrium diagram (VLE) of the system CO_2-organic solvent depends on the conditions of temperature, pressure and on Gas to Liquid Ratio of the Expanded Liquid (GLR-EL, on mass basis). Gas to Liquid Ratio of the Expanded Liquid of 1.8, with a CO_2 flow rate of 12 g/min was used for all the experiments. Other fixed parameters: saturator temperature 80 °C, pressure 95 bar, suspension recovery at room conditions, solvent/antisolvent ratio 1/8. The solution at the exit of the saturator is injected into the receiving water phase using an orifice that has a diameter of 100 μm. The receiving phase is formed by water in which the solute is not soluble containing Tween 80 (0.2 % w/w), used as surfactant.

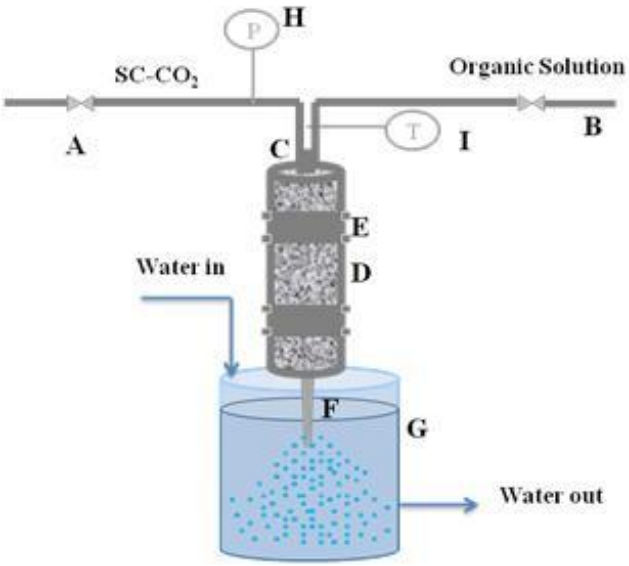


Figure 1. Detail of the saturation-injection of the SAILA process apparatus

2.3 SLNs characterizations

Dynamic Light Scattering (DLS) technique (mod. Zetasizer Nano S, Worcestershire, UK) was used to obtain mean diameter and particle size distribution (PSD) of Solid Lipid Nanoparticles produced with SAILA. No dilution of samples were performed. Each measurement was repeated in triplicates. The same instrument was also employed to measure surface zeta potential of the produced vesicles.

Dynamic laser scattering was also used to observe particles stability in water suspensions measuring SLNs mean diameter at regular time interval, over a period of 30 days.

Nanoparticle 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 concentrated nanoparticle dispersions over aluminium stubs and drying them at air for 2 days. The dried samples were then coated with a gold layer with a sputter coater (thickness of 250 Å, model B7341, Agar Scientific, Stansted, U.K.).

3. Results and discussion

SLNs production experiments using the SAILA process were performed with different lipids: soy lecithin, cholesterol and stearic acid. In particular, the effect of lipid concentration in the solvent solution on particle size distribution of the obtained suspensions was investigated.

Soy lecithin SLNs

In the first set of experiments, soy lecithin was dissolved in ethanol. Lipid concentration was varied from 5 to 40 mg/mL.

Table 1. Type and concentration of lipids and solvent for SAILA SLNs production experiments. Mean Diameters (MD) and Standard Deviations (SD) of Solid Lipid Nanoparticles (SLN) produced. *These experiments were performed with the addition of surfactants.

Lipid	Concentration [mg/mL]	Solvent employed	MD ± SD [nm]
Soy lecithin	5	Ethanol	158 ± 53
	10		159 ± 46
	20		233 ± 85
	30		216 ± 120
	40		326 ± 169
Stearic acid	5	Acetone	*Not spherical*
	5*****		364 ± 77
	10*****		462 ± 88
Cholesterol	5	Acetone	*Not spherical*
	5*****		151 ± 74
	10*****		207 ± 57

Soy lecithin SLNs were successfully produced; indeed, there was no need to add a surfactant for this set of experiments. Increasing the lipid concentration in the solvent solution, stable nanoparticles suspensions were obtained. In particular, increasing soy lecithin concentration from 5 to 40 mg/mL in ethanol solution, an overall effect of SLNs mean diameter increase and enlargment of the PSD was observed, as reported in **Table 1**. In details, mean diameters increased from 158 ± 53 nm obtained operating at a concentration of 5 mg/mL to 326 ± 169 nm produced using the highest concentration of 40 mg/mL.

In particular, the overall effect of soy lecithin concentration in ethanol on SLNs PSD was graphically represented in **Figure 2**, where the enlargement of the PSD with the increase of the lipid concentration is well visible.

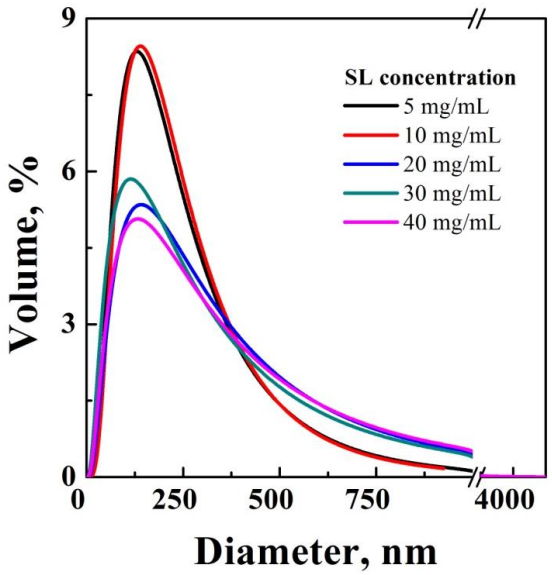
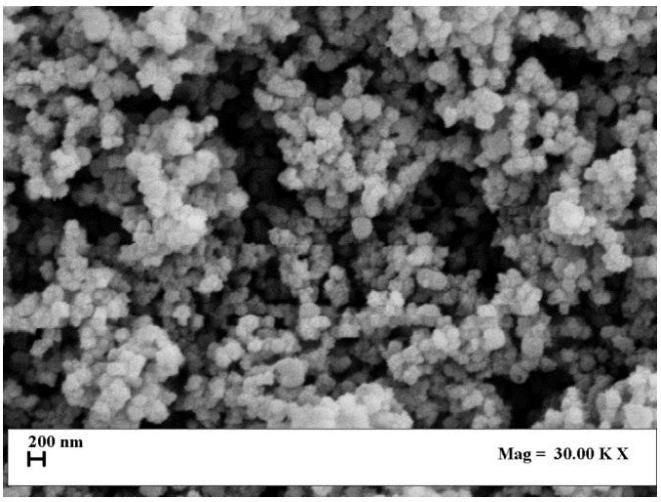


Figure 2. Effect of soy lecithin concentration on SLNs particle size distributions

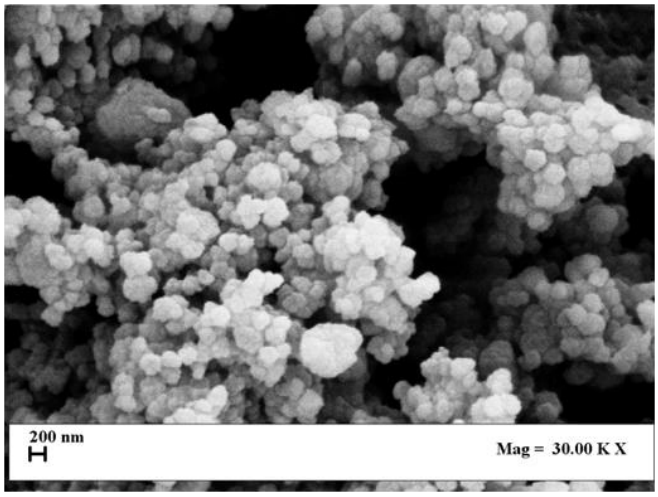
Lipid concentration in SAILA feeding solution has influenced the extension of particle size distributions and mean diameter of particles obtained. In particular, an increase of polidispersion indexes (see **Table 1**) was observed for the sample produced at concentrations higher than 20 mg/mL, whereas the samples obtained at 5 and 10 mg/mL presented particles size distributions practically overlapping.

This result can be explained considering the nucleation process and its dependence on the concentration of the solute: a higher supersaturation favors particles growth; therefore, increasing the solute concentration, larger particles are obtained.

FE-SEM images of particles obtained at 20 mg/mL and 40 mg/mL are reported in **Figure 3ab.**



(a)



(b)

Figure 3. FE-SEM image of soy lecithin SLNs obtained at 20 mg/mL (a) and 40 mg/mL (b) solution concentration

From these figures, it is possible to see that particles appear irregularly spherical with dimension larger than the reference bar (200 nm), confirming data reported in **Table 1**.
Zeta potential of Soy Lecithin samples was about -0.5 mV for all the experiments, independently on the increase of lipid concentration.

Stearic acid SLNs
The production of SLNs was attempted with stearic acid (see **Table 1**). Since this lipid is not soluble in ethanol, acetone was used as solvent. The same process conditions adopted for soy lecithin were employed for this new set of experiments.

The first experiment, performed at 5 mg/mL of lipid concentration in acetone solution, was not successful since not spherical particles were obtained, as reported in the FE-SEM image of **Figure 4**.

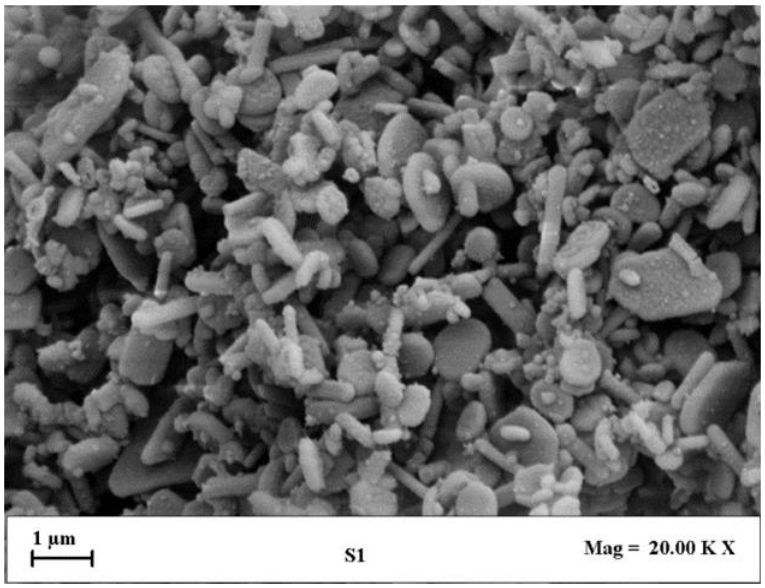


Figure 4. FE-SEM image of stearic acid particles obtained at 5 mg/mL of lipid concentration in acetone solution

As shown in this figure, obtained particles were characterized by not regular and controlled shape; furthermore, the suspension was not stable and stearic acid formed aggregates that precipitated on the bottom of the vessel. An uncontrolled crystallization of stearic acid was probably the cause of this result.

Another experiment was performed adding a surfactant in the lipid solution. For this reason, 0.2 % w/w of Span 20 was added, as a cosurfactant, to the acetone lipidic solution, since it is reported in literature that the addition of this lipophilic surfactant, coupled with the hydrophilic surfactant of the antisolvent, can improve suspension stability against sedimentation, flocculation and coalescence. All other SAILA process parameters were maintained constant.

The addition of surfactants in the antisolvent and solvent phases resulted in the successful production of SLNs with SAILA process. Stable and homogeneous lipidic nanoparticles in water suspensions were obtained with mean diameter of 364 ± 77 nm, as it is possible to see from **Table 1**. Stearic acid concentration in acetone solution was increased to 10 mg/mL and an increase in the mean diameter of produced particles was observed in this case. SLNs with a mean diameter of 462 ± 88 nm were obtained. The increasing mean diameter trend was confirmed increasing lipid concentration. A FE-SEM image of stearic acid nanoparticles obtained using the SAILA process is reported, as an example, in **Figure 5**.

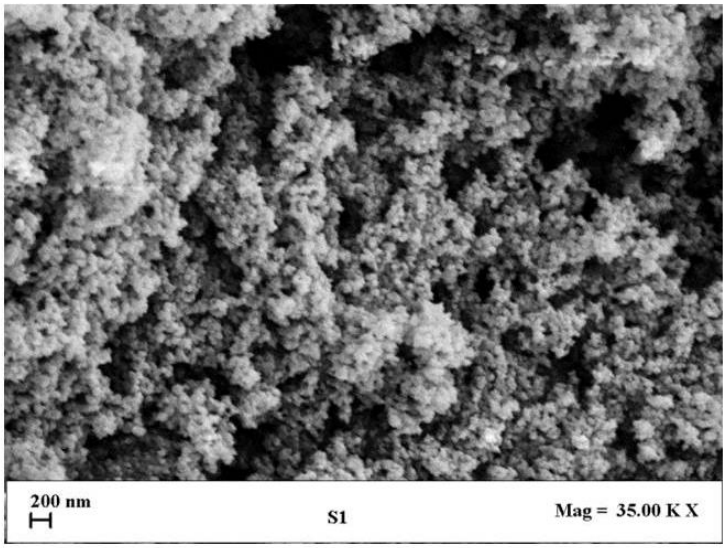


Figure 5. FE-SEM image of stearic acid particles obtained at 5 mg/mL of lipid concentration in acetone solution with the addition of Span 20 surfactant in acetone solution

From FE-SEM observation, it is possible to affirm that the addition of surfactants modified the morphology of nanoparticles; SLNs are approximately spherical; this was probably due to the presence of surfactants, that stopped the process of stearic acid crystallization. From microscopic observations, nanometric mean dimensions are confirmed for both lipid concentrations.

Particles size distributions of stearic acid nanoparticle suspensions produced at different lipid concentrations in acetone solution are compared in **Figure 6a-b**.

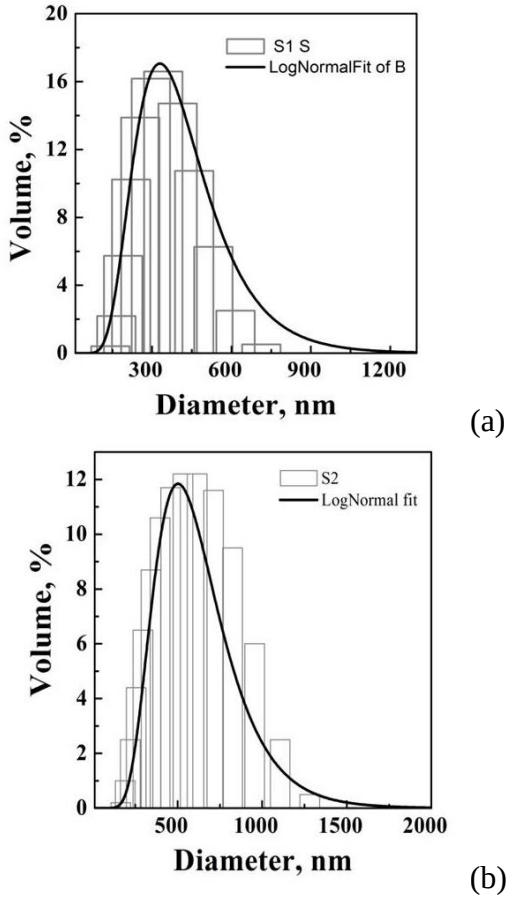


Figure 6 Particle Size Distributions of stearic acid SLNs obtained at 5 mg/mL (a) and 10 mg/mL (b) of lipid concentration in the solvent solution

Observing the results shown in **Figure 6** and looking at data of **Table 1**, it is possible to note that particles obtained with a stearic acid concentration of 5 mg/mL showed a mean diameter of 364 ± 77 nm, considerably larger than mean diameter obtained using soy lecithin as lipid at the same process conditions. Also in this case, increasing lipid concentration, an increase of SLNs dimension was obtained. Indeed, SLNs with a mean diameter of 462 ± 88 nm were produced with the higher stearic acid concentration of 10 mg/mL. Doubling the concentration of lipid dissolved in acetone, particle size distributions showed a similar amplitude, but mean size of samples was increased.
Surface zeta potential was measured for stearic acid SLNs. Sample characterized by irregular shape, produced without surfactants, has a surface charge of -18.5 mV; whereas, the samples produced with surfactants have a potential of -4.72 mV for 5 mg/mL stearic acid concentration and -3.68 mV for 10 mg/mL. SLNs surface charge was modified by the addition of the second surfactant; probably, non ionic surfactants employed in these two experiments contributed to partially neutralize the starting surface charge of nanoparticles.

Cholesterol SLNs
The production of cholesterol SLNs was also attempted and the effect of cholesterol concentration on diameter and morphology of the obtained SLNs suspension was studied in analogy with the other lipids investigated in this work. Since cholesterol is slightly soluble in ethanol, also in this case acetone was used for the preparation of the solvent phase.
The first experiment was performed with cholesterol concentration of 5 mg/mL in acetone, without the use of a surfactant. FE-SEM image of this sample was reported in **Figure 7**.

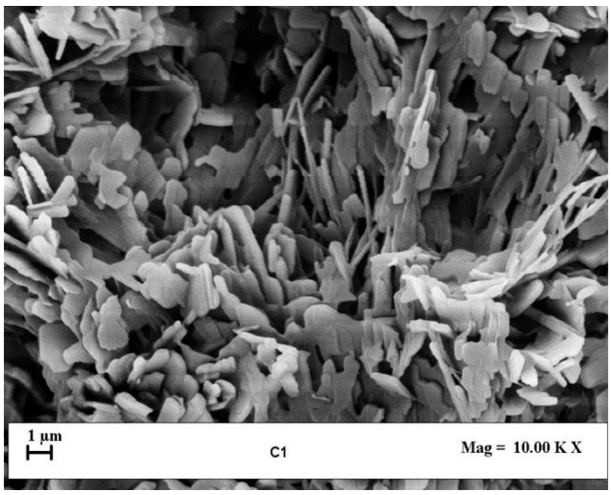
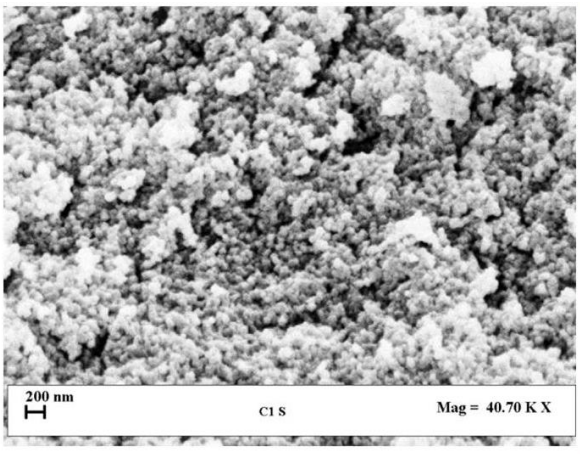


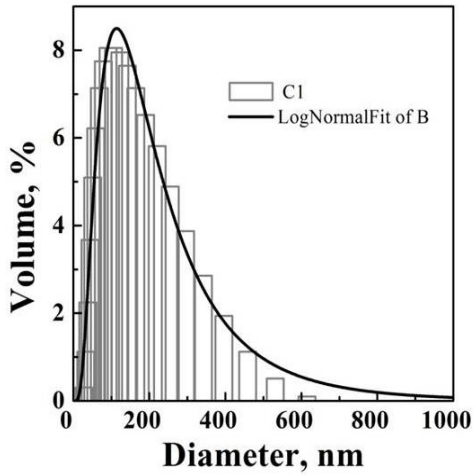
Figure 7. FE-SEM image of cholesterol crystals obtained without the use of a surfactant for SLNs production

The first experiment was unsuccessful. As obtained for stearic acid, the absence of a surfactant caused the production of instable SLNs, characterized by aggregation tendency. As also reported in literature, it is difficult to control cholesterol crystallization during particles formation [47]. From FE-SEM observation, plane and irregular micrometric particles were obtained. This behaviour was probably caused by fast and uncontrolled crystallization phenomena of cholesterol in the water environment. For this reason, also in this case, the couple of lipophilic and hydrophilic surfactants were used respectively in the solvent and anti-solvent phase to obtain a better control of particle precipitation process.

Adding Span 20 at the same concentration used for stearic acid experiments, a stable suspension was recovered after the process and particles with a mean diameter of 151 ± 74 nm were obtained. A FE-SEM image of SAILA cholesterol SLNs produced at 5 mg/mL has been reported in **Figure 8a**, together with the relative particle size distribution (**Figure 8b**).



(a)



(b)

Figure 8. FE-SEM image of cholesterol SLNs obtained at 5 mg/mL (a) with Particle Size Distributions (b)

Particles appear spherical and irregular and zeta potential was -4.50 mV, in the same range of value of the surface charge of stearic acid nanoparticles; also in the case of cholesterol the addition of the lipophilic surfactant allowed a better control of particles precipitation process and the production of nanoparticles was possible. Without the second surfactant only micrometric crystals were recovered. Increasing Cholesterol concentration to 10 mg/mL, larger particles with a mean diameter of 207 ± 57 nm were obtained. Doubling the concentration of cholesterol, SLNs increased their mean diameter, as obtained for stearic acid and soy lecithin nanoparticles. Surface zeta potential of samples with 10 mg/mL cholesterol concentration was -4.05 mV, similar to the previous obtained for 5 mg/mL concentration in acetone.

A comparison of PSDs obtained for the three processed lipids at the same concentration of 5 mg/mL is reported in **Figure 9**.

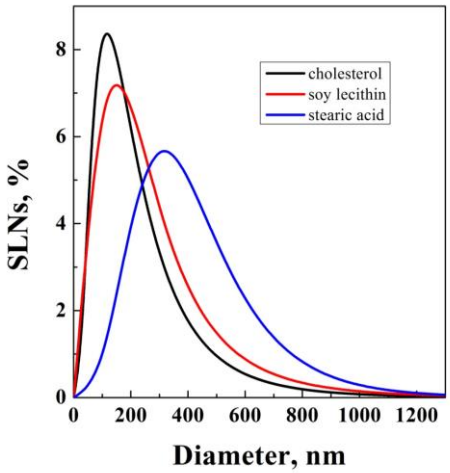


Figure 5 Soy lecithin, cholesterol and stearic acid SLNs particle size distributions produced with SAILA process t 5 mg/mL of lipid concentration in the solvent solution

Cholesterol and soy lecithin show approximately the same results; whereas, larger particles were obtained in the case of stearic acid.

A study on the stability of soy lecithin, cholesterol and stearic acid SLNs was performed measuring the mean diameter of the obtained suspension, during 30 days of storage at 4 °C.

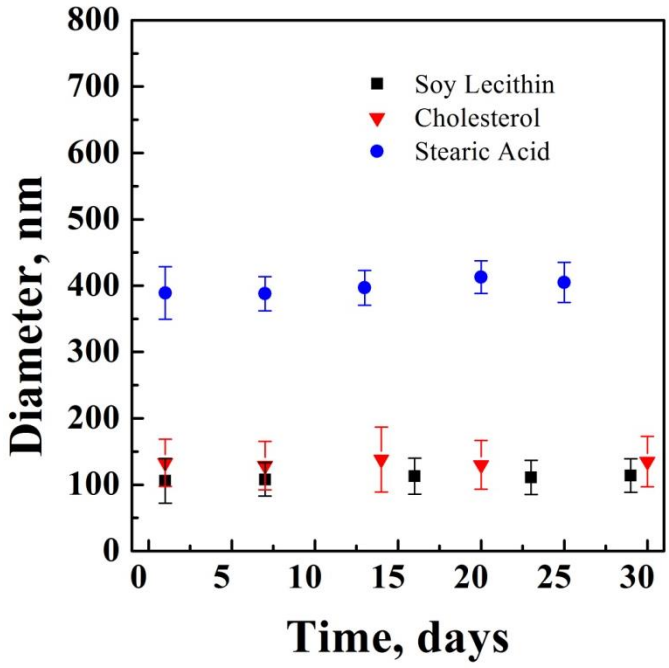


Figure 10. Storage stability of SLNs of different lipids produced using the SAILA process

As shown in **Figure 10**, SLNs suspensions were almost stable over the full time of observation. This result is explainable considering the stable values of surface charge of these particles that allowed a high steric repulsions between particles in suspension, avoiding aggregation during time. Stability of soy lecithin SLNs was obtained without the addition of surfactants since it is characterized by an amphipathic molecular structure, that makes it behave as a natural emulsifying agent. Whereas, the stability of stearic acid and cholesterol SLNs was due to the presence of surfactants Span 20 and Tween 80.

4. Conclusions

In this work, SAILA process was successfully employed for the production of solid lipid nanoparticles, using lipids of different nature such as soy lecithin, stearic acid and cholesterol. For all the cases observed, an increase of particles mean diameter was obtained increasing lipid concentration in the solvent solution. A dependence of SLNs mean diameter on lipid concentration in the expanded liquid solution was observed: a higher supersaturation favors particles growth. An important synergistic effect of lipophilic and hydrophilic surfactant was observed and it revealed crucial to control the precipitation of particles for cholesterol and stearic acid with high tendency to fast and uncontrolled crystal growth.
Future perspectives of this work will regard the possibility to study other SAILA process parameters to further optimize SLNs production for the entrapment of drugs inside the lipid carriers.

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