PRODUCTION OF COPPER LOADED LIPID MICROPARTICLES BY PGSS ®

(PARTICLES FROM GAS SATURED SOLUTIONS) PROCESS

Víctor Martín¹, Vanessa Gonçalves¹,²,³, Soraya Rodríguez-Rojo¹*, Daniela Nunes⁴, Elvira Fortunato⁴, Rodrigo Martins¹, María José Cocero¹, Catarina Duarte²,³

¹High Pressure Processes Group, Department of Chemical Engineering and Environmental Technology, School of Engineering, Venue Dr. Mergelina, University of Valladolid, Dr. Mergelina s/n, 47011, Valladolid, Spain

²Instituto de Tecnologia Química e Biológica António Xavier, Universidade NOVA de Lisboa, Avenida da República, 2780-157 Oeiras, Portugal

³Instituto de Biologia Experimental e Tecnológica, Apartado 12, 2781-901 Oeiras, Portugal

⁴i3N/CENIMAT, Department of Materials Science, Faculty of Sciences and Technology, Universidade NOVA de Lisboa, Campus de Caparica, 2829-516 Caparica, Portugal

*Corresponding author:

E-mail address: sorayarr@iq.uva.es
Abstract

Production of lipid particles loaded with metal nanoparticles by supercritical fluids based processes has been barely studied. In this work, copper nanoparticles were loaded into glyceryl palmitostearate microparticles by PGSS® (Particles from Gas Saturated Solutions). The effect of different variables, temperature (60-80 °C), copper load (0.2-5% w/w) and water addition (0 – 40% w/w), in particle size and encapsulation efficiency has been studied. The dispersion of metal nanoparticles in the lipid has been determined by SEM-FIB coupled with EDS mapping. In all cases, mean particle size values lower than 70 μm have been obtained, and encapsulation efficiencies around 60% have been achieved. The addition of water has no negative effect in encapsulation efficiency nor in nanoparticles dispersion within the lipid microparticle, being important since nanoparticles are commonly synthetized in aqueous medium.

Keywords: PGSS®; copper nanoparticles encapsulation; lipid microparticles; dispersion
Nanoparticles, especially noble metal nanoparticles, have an emergent importance in biomedicine field. Their uses are diverse, for example, in molecular imaging, targeted drug delivery systems, targeted therapies (hyperthermia, gene silencing or radiotherapy), and biosensors. These wide applications are possible thanks to nanoparticle properties such as specific area, superior narrow range of emission, photo stability, broad excitation wavelength, quantum dots and the possibility of being functionalized [1, 2].

One of the most interesting metals is copper. This transition metal has biological activity as anti-inflammatory, anti-proliferative, and biocidal agent, and present some radioisotopes useful for nuclear imaging and radiotherapy [3]. On the other hand, copper organometallic complexes can be used to deliver copper ions or radionuclides to diseased tissues or to modify pharmacokinetics. These copper compounds can be managed by organism since copper is an essential microelement in contrary to other transition metals. For example, copper (II) complexes have anti-inflammatory and anti-proliferative properties and thus could be used in chemotherapy. Moreover, copper in metallic form possesses antimicrobial activity, already used in agriculture. It can degrade DNA by mean of the generation singlet oxygen [4], therefore, it is studied as anti-cancer and anti-proliferative agent [3, 5, 6].

In order to apply copper nanoparticles for biomedical applications it is necessary to encapsulate them in order to protect the metal until it arrives to the desired zone, to avoid the damage in healthy cells owing to their cytotoxicity. Since lipids are well tolerated by human body and have low toxicity, they are adequate carriers for the encapsulation of metal nanoparticles. Besides, they present advantages over other
colloidal carriers in terms of active compound stability and protection, being possible to be administered in inhalable, transdermal, intravenous or oral form [7].

Conventional methods for producing lipid microparticles are microemulsions or double emulsions followed by spray drying or spray chilling [8, 9]. However, these methods involve the use of organic solvents, severe operation conditions and purification steps. PGSS® (Particles from Gas Saturated Solutions) is a technique with the capacity of avoiding conventional technic mentioned drawbacks [10]. In this process, the lipid is melted with the dissolved or suspended active compound, and the final mixture saturated with supercritical carbon dioxide. Then, this suspension is expanded through a nozzle into an expansion chamber and fine copper lipid coated particles are formed [7, 11, 12]. One of the advantages of PGSS® in relation to other supercritical fluid technologies is that the substance does not need to be soluble in carbon dioxide, like in the case of Rapid expansion of supercritical solutions (RESS). The production of lipid nanoparticles loaded with metal has been barely studied with this process. Up to the authors knowledge only the group of Bertucco worked on the production of lipid microparticles magnetically active with excellent results using triestearin, phosphatidylcholine and magnetite nanoparticles [13]. In contrast, there are studies about processes in which a polymeric matrix is used in spite of lipid. These processes are based on emulsion technology (microemulsions, miniemulsions, double emulsions) [14, 15]. Further, this process have been combined with supercritical fluid technology for the elimination of the solvent as in the production of, poly (lactic-co-glycolic) acid (PLGA) nanoparticle loaded with magnetite have been formulated by means of supercritical fluid extraction of emulsions [16].

In this work, a study of PGSS® process to obtain copper lipid loaded microparticles was performed. The operation conditions were chosen regarding the nature of the lipid
used and the variation of its properties when in contact with supercritical carbon dioxide and its influence on the physical properties of the precipitated particles was investigated. Moreover, the effect of metallic nanoparticle load in the product and encapsulation efficiency were studied in order to establish an operational limit. Finally, and since nanoparticles are usually obtained in aqueous dispersion [17], the effect of water in the dispersion of metal in the lipid matrix and particle morphology was observed.

2. Materials and methods

2.1 Materials

Precirol® ATO 5 (glyceryl palmitostearate) was kindly supplied by Gattefossé (France). Imwitor® 600 was supplied by Sasol (Germany). Carbon dioxide with 99.95 mol% purity was delivered by Air Liquide (Portugal). Copper nanoparticles were purchased from Alfa Aesar with a particle size of 20 to 30 nm. All the chemicals have been used without further purification.

2.2 Precipitation of copper loaded lipid particles by particles from gas saturated solutions (PGSS®)

In order to produce the loaded particles, Precirol 5 ATO is placed in a 50 cm³ high pressure stirred vessel, electrically thermostated at the selected operation temperature. Then, the required amount of copper nanoparticles are added. In the experiments carried out with water, the necessary amount of water and 3 mg of Imwitor® 600 (HLB = 4) are incorporated. Imwitor® is a water/oil emulsifier that is necessary to form a macroemulsion, since Precirol has low hydrophilic lipophilic balance (HLB = 2) [11]. In this case, it resulted in an macroemulsion. Thereafter, the vessel is closed and the
mixture stirring (150 rpm) begins. Carbon dioxide is pumped by a high pressure pneumatic piston pump to the vessel until experimental pressure is achieved. The mixture and the supercritical carbon dioxide are brought into contact during 15 minutes, since no pressure depression was observed after this period being the ideal mixing time [11]. Then, the stirred mixture is depressurized through a nozzle (250 µm) by means of an automated valve to expansion chamber. In this chamber access, the expanded suspension is mixed with compressed air (0.7 MPa, 25ºC) for improving drying. The particles are collected in an 18 L container. The equipment flow diagram can be seen in figure 1.

(FIGURE 1)

Some experiments were performed previously to fix the pressure conditions in the pre-expansion chamber. A value of 10 MPa was selected since an increase in the pressure (up to 15 MPa) did not reduce the particle size, varying also the mixing temperature between the studied range, from 60ºC to 80ºC. The variables studied apart from temperature were the copper load, from 0.2 to 5%, and the addition of water from 0 to 40% of the mass of copper and lipid. Random experiments were repeated showing the good reproducibility of the process.

2.3 Particle characterization

Particles have been characterized regarding their size distribution, morphology and metal dispersion in the lipid matrix.

2.3.1 Particle size distribution of lipid loaded microparticles

Particle size distribution was measured by laser diffraction using a Mastersizer 2000 (Malvern Instruments) with red light (max. 4 mW helium-neon, 632.8 nm). This
equipment has an accuracy and a reproducibility better than 1%. The particles were dispersed in water with surfactant (Pluronic) to improve the dispersion due to the Precirol 5 ATO low HLB. The results are expressed as particle volume distribution average diameter ($d_{0.5}$) and spam. Average diameter and spam values are an average from three different measurements. Spam is defined as the ratio between the $d_{0.5}$ and the difference between $d_{0.9}$ and $d_{0.1}$. If the value is near to 1, the particle size distribution is narrow. Precirol refractive index selected was a generic lipid index (1.6).

2.3.2 Morphology and metal dispersion

Particle morphology and copper metallic nanoparticles dispersion in the lipid matrix were analyzed by scanning electron microscopy (SEM). Images were taken by a JEOL JSM-820, 20 kV, 23-mm working distance at vacuum conditions equipment. Previous to the analysis, the samples were coated with gold in an argon atmosphere. Furthermore, particles were studied through Focused Ion Beam (FIB) couple to SEM using a Carl Zeiss AURIGA CrossBeam workstation instrument, equipped with an Oxford EDS spectrometer. The particles were dispersed in carbon tape and covered with an Au/Pd conductive film. Ga$^+$ ions were accelerated to 30 kV at 50 pA. The etching depth is around 0.2 μm.

2.4 Chemical characterization

The metal load in the particle has been analyzed by inductive coupling plasma with optic emission spectrometry technic (ICP-OES). It was performed with an atomic emission spectrophotometer ICP-OES Varian 725-ES using argon as carrier gas. The samples were digest with nitric acid in a microwave oven in order to oxidize copper to ionic state and eliminate the lipid. The results are expressed as mg of copper per gram.
of lipid. The method has an error in calibration lower than 2%. Some samples were random repeated in order to check the repeatability of process.

Encapsulation efficiency has been calculated from metal load data, as it can be seen in equation 1. $C_0$ is the theoretical concentration, the product introduced in the process, while $C_i$ is the real concentration measured by ICP.

\[
\% \text{ encapsulation efficiency} = \frac{C_i}{C_0} \times 100
\]  

ICP chemical analysis was confirmed by TGA showing similar data in all the cases with a difference between both methods lower than 7%, additionally the amount of water in the final encapsulated product was obtained. The equipment utilized was TGA/SDTA RSI analyzer of Mettler Toledo. Samples of approximately 10 mg were heated from 50 °C to 600 °C at a rate of 20 °C/min under N\(_2\) atmosphere (60 N mL/min flow). Water loss was taken into account from 25 °C to 120 °C.

3. Results and discussion

Before experiments were performed, it was verified that the nanoparticles do not significantly agglomerate when mixing with the carrier material, Precirol, to assure the viability of the process. The mean agglomerate size, as volumetric d0.5, was below 100nm, as shown in Figure 1S.

Main experimental results are summarized in table 1.

(TABLE 1)

3.1 Effect of temperature and pressure conditions

The selection of range of temperatures used in this work was made in accordance with the lipid melting point variation in the presence of carbon dioxide, studied by A.R.S. de Sousa et al. [18]. The authors verified that the melting point reduces from 63 °C to 50
°C, when pressure increases from ambient to 10 MPa, then, the value remains almost constant up to 30 MPa. For this reason, the range of mixing temperature tested has been between 60 °C and 80 °C; the reduction of mixture viscosity improves the atomization of the molten into smaller particles in the depressurization step: increasing the temperature decreases the viscosity of the lipid molten it-self, and lower temperatures increase solubility of carbon dioxide reducing also the viscosity [17][19]. On the other way, it also influences the cooling and solidification rate in the expansion chamber [19], thus smaller particles are expected due to less droplet coalescence if mixing temperature is close to the melting point of the processed material, the lipid in this case.

(FIGURE 2)

Concerning particle morphology, it is important to observe that the temperature does not have any effect, as it can be seen in figure 2. The same flaked morphology remains when the temperature increased (Images A and B). Similarly, the final particle size does not experiment changes. Figure 3 shows two particle size distributions at different values of temperature, maintaining the other parameters constant. The two distributions are almost identical, meaning that particle formation is almost unaffected by temperature in this experimental conditions due to a counter balance of its influence in the viscosity and cooling rate of the molten, previously discussed.

(FIGURE 3)

In conclusion, in the studied range conditions, temperature has not effect in the final product morphology and size but it has an obvious effect in the encapsulation. In the experiment 2 at 60 °C, encapsulation efficiency is higher than in experiment 3 at 80 °C. For these reasons, the experiments were performed with the lower values of pressure
and temperature (60 °C and 10 MPa), at these conditions, the solubility of carbon
dioxide in the lipid is 0.23 g CO₂/g lipid.

3.2 Influence of metal dispersion and metal load

Microparticles morphology have been analyzed by SEM and SEM-FIB technique. This
flaked microparticles (Figure 2) present big hollows irregularly distributed, as shown in
the FIB-cut images (Figure 4), and nanoparticles are present in the thin lipid
membranes, which define these light structures.

The dispersion of copper nanoparticles in the lipid matrix has been measured by EDS
mapping of the FIB-cut images (Figure 4).

(FIGURE 4)

In the experiment with 0.2% of copper without water at 60 °C and 10 MPa (figure 4.a),
it can be seen over the particle an homogeneous dispersion of copper because of the red
color is in the particle contour uniformly, while in the other three images (4b and 4c),
which have 5% of copper, it can be observed tiny particles agglomerations. We can
conclude that high copper loading promotes higher degree of nanoparticle
agglomeration and hence, worse dispersion of the metal is achieved. Regarding the
influence of temperature, comparing figure 4b (60°C) and 4d (80°C) there is no
substantial differences between them, although there are not copper agglomerates in the
image 4d, as in figure 4a for low copper load due to the lower encapsulation efficiency
observed at higher temperature. Also, nanoparticles are slightly better dispersed when
the temperature is increased due to the reduction in viscosity in the pre-expansion
mixture. Finally, water has not a significant effect in the dispersion, as can be noticed
when figures 4b and 4c are compared. This is important since nanoparticles are often
produced as aqueous dispersions, and their use as raw material will not affect the
process performance with respect to the use of powder nanoparticles.

Different values of copper mass were tested. This parameter was varied from 0.007 to
0.150 grams, maintaining the total amount in the chamber of 3.000 grams. These values
correspond with a theoretical load from 0.2 to 5%. As it can be seen in figure 5, the
general trend is that the encapsulation efficiency increases as the mass of copper
increases, as expected there is more copper available to be encapsulated; Besides, the
powder agglomeration in the pre-expansion mixture is increased, as previously
indicated. It can be noticed that at 0.2% copper load the value of encapsulation
efficiency is unusually high; this effect has been observed by other researchers when the
amount of material to be encapsulated is very low [20], since it is statistically more
probable to be all encapsulated.

(Figure 5)

Regarding particle size, there are not significantly differences associated to mass copper
variation (Table 1. experiments 2, 3 and 9) obtaining values between 43 and 49 μm,
unless the experiment with the minimum efficiency, which presents a minor size (33
μm).

3.3 Initial water influence

In numerous processes, it is possible to obtain nanoparticles in aqueous suspension.
Thus, trying to reduce separation steps necessary to obtain solid nanoparticles. PGSS®
process has been proved with different amounts of water to study the effect in the
dispersion and micronization processes.

Water content was varied from 0 to 40% to determine its effect in process performance
at two different copper loads maintaining the other parameter constant. As previously
mentioned, there is no significant effect of water addition in nanoparticles dispersion (Figure 4). Similarly, there is not a significant effect on encapsulation efficiency (figure 6) at 5% Cu load, probably due to the fact that the water remains as independent phase and the nanoparticles have affinity by the lipid phase. In table 1 data for experiments carried out at 0.2% copper load is also presented, and in general the same trend is observed, although as commented before due to the small amount present they maybe not fully representative.

(Figure 6)

Similarly, particle size behavior does not present differences regarding water addition to the initial mixture as it can be seen in table 1.

(Figure 7)

Regarding particle morphology, water presence makes flaked particles to be more compact as it can be seen comparing figure 8 with figure 2. Probably due to the longer time required for particle surface solidification that promote the amalgamation of flakes.

(Figure 8)

Finally, through thermogravimetric analysis it is possible to know that the amount of water in the final encapsulate product is below 0.7% for the experiments with the higher amount of water. This is a good result since it indicates that almost all the water is eliminated in expansion process.
4. Conclusions

This work is a preliminary study proving that metal nanoparticles can be successfully incorporated in lipid microparticles by PGSS® process. This is a one-step green process that involves the use of carbon dioxide as unique external agent to generate the particles in the micrometric range from a molten mixture.

In the tested range of operating conditions (P = 10 MPa and T = 60-80°C), it has been concluded that the main process parameter is copper content (%). When copper load is augmented the encapsulation efficiency increases without an important influence in particle size, although the metal nanoparticles, which are in general uniformly distributed in the lipid, tends to form small agglomerates.

Since metal nanoparticles can be produced as aqueous suspensions, the effect of water addition (up to 40% w/w) has been studied showing no significant effect in encapsulation efficiency nor in nanoparticles dispersion within the lipid microparticle.
Acknowledgments

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References


20. R. Couto, V. Alvarez and F. Temelli (2016) *Encapsulation of Vitamin B2 in solid lipid nanoparticles using supercritical CO₂*. J. of Supercritical Fluids, DOI: http://dx.doi.org/10.1016/j.supflu.2016.05.036.
Figure 1 Experimental setup FAME Separex: (1) carbon dioxide cylinder, (2) cryostate, (3) pneumatic pump, (4) stirred vessel, (5) depressurization valve, (6) cyclone and (7) nozzle d=250 µm, with external mixture with compressed air.
Figure 2: SEM micrographs of copper-lipid particles produced by PGSS®. Particles in A at 60 °C and 10 MPa (Exp. 2, table 1) and particles in picture B at 80 °C and 10 MPa (Exp. 3, table 1).
Figure 3: Particle size distributions in volume at various temperature conditions (copper load 0.2%, water amount near 30%) (Exp. 2 and 3, table 1).
Figure 4: SEM-FIB analysis of copper loaded lipid particles with EDS mapping (blue-carbon and red-copper): A-0.2% Cu, T = 60 °C, P = 10 MPa, initial water content = 0%, B- 5% Cu, T=60 °C, P= 10 MPa, initial water content = 0%, C- 5% Cu, T=60 °C, P= 10 MPa, initial water content = 40% and D- 5% Cu, T=80 °C, P= 10 MPa, initial water content = 0
Figure 5: Copper mass influence in encapsulation efficiency.
Figure 6: Variation of encapsulation efficiency with initial content of water (Pressure 10 MPa, temperature 60 °C, 5% Cu load)
Figure 7: Variation of final product particle size with initial content of water (Pressure 10 MPa, temperature 60 °C, 5% Cu load)
Figure 8: Effect of water in morphology. SEM image from experiment 4 (temperature 60 °C, pressure 10 MPa, copper load 5% and water content 20%).
Table 1: PGSS experiment list. En las figuras muestras barras de error para encapsulación y para tamaño de partícula, aquí deberían aparecer valores con +/-.

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<th>Experiment</th>
<th>Precirol (g)</th>
<th>Copper load (w/w%)</th>
<th>Water content* (w/w%)</th>
<th>Temperature (ºC)</th>
<th>Encapsulation efficiency (w/w%)</th>
<th>Particle size $d_{0.5}$ (µm)</th>
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* Water content respect lipid + copper mass.

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<th>Experiment</th>
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Supplementary Information

The particle size of the copper nanoparticles dispersed in different media was measured using a dynamic light scattering (DLS) analyzer with a He-Ne laser of 633nm (Zetasizer Nano ZS Malvern Instruments Ltd., Malvern, UK). Dispersions of the necessary amount of copper were prepared using a laboratory magnetic stirrer in water with pluronic F127 as surfactant to improve the wettability of the nanoparticles, and the carriers employed in the PGSS experiments: precirol melted at 60°C and precirol melted with water and imwitor. For each dispersion, about 0.5-1 mL was introduced into the Zetasizer using a disposable cuvette, and particle size was measured 3 times in the DLS equipment and analyzed in duplicate (independent samples). For the samples including precirol the temperature of 60°C was maintained through all the measurement time (ca. 10 min). The volume distribution was used to find out the d0.5 of the particles. Figure 1S shows the results as mean value of the six measurements per sample and standard deviation.

Mean agglomerate size in precirol and precirol + 40% water is 70 +/- 20 nm and 40 +/- 30 nm, respectively, which is significantly lower from the value measured in water with a surfactant (600 +/- 240 nm) were the nanoparticles form big agglomerates and tend to sediment. These results show the affinity of the nanoparticles for the lipid phase.

![Figure 1S](image-url)

**Figure 1S.** Nanoparticles agglomerates size (volumetric d0.5) dispersed in different media.