Supercritical fluid extraction of emulsions to produce biopolymers microparticle and nanoparticle

Campardelli R.*[(a)], Della Porta G.[(a,b)], Reverchon E.[(a)]

(a) Dipartimento di Ingegneria Industriale, Università degli Studi di Salerno. Via Ponte Don Melillo, 84084 Fisciano (SA), Italy.

(b) Laboratorio di Ingegneria Cellulare e Molecolare, DEIS (sede di Cesena), Università di Bologna, Via Venezia 52, Cesena (FC), Italy.

*corresponding author: rcampardelli@unisa.it

Abstract

Supercritical fluids extraction of emulsions (SEE) is an innovative technique used for the production of micro, nano-particles with controlled size and distribution. Supercritical carbon dioxide (SC-CO_2) is used to extract selectively the organic solvents from the droplets of the emulsion, in which the biopolymer is dissolved, producing the precipitation of the polymer and the subsequent formation of particles. In the SEE technology, the dimensions of the desired polymeric particles are directly connected to the dimensions of the droplets in the emulsions, indeed changing the emulsion formulation parameters it is possible to vary the dimensions of the particles produced after the solvent removal. SEE technology was also developed in continuous operation layout (SEE-C) to improve the product recovery and processing. In our previous work, SEE-C technology was successfully applied to produce microparticles starting from a new emulsion preparation method using acetone as the solvent of the dispersed phase in place of common used solvents (ethyl acetate, methylene chloride, chloroform). Only particles in the micrometric range of dimensions were produced by SEE-C technology starting from this new emulsion formulation. The aim of this work is to try to establish the conditions at which by SEE-C is possible to vary the dimensions of the particles form micro size range to nano size range. This work focuses on particle engineering from microparticles to nanoparticles for different biopolymers, changing the formulation of the emulsion using acetone as disperse solvent. Varying the emulsion formulation parameters such as, surfactant and biopolymer amount, phases ratio and emulsification technique (ultrasound or high speed emulsification), droplets dimensions have been changed from the microsize to the nanosize range, allowing the production of micro and nanoparticles of PMMA (poly-metylmetacrylate) and PCL (poly-caprolactone) of desired size and distribution after supercritical solvent extraction.

INTRODUCTION

Biodegradable polymers are mainly used where the transient existence of materials is required [1]. Biodegradable particulate systems are interesting for controlled drug release [2], drug targeting [3], but also as support matrices in injectable scaffold formulation for the regenerative medicine [4]. The main used biopolymers in these fields are poly-lactic acid (PLA) [5] and poly-actic-co-glycolic acid (PLGA) [6].

The success of these for pharmaceutical applications has further led to the evaluation of other kind of polymer such as poly-ε-caprolactone (PCL) and poly(methyl methacrylate) PMMA for the production of micro and nanospheres. For example, the advantages of PCL use include its high permeability to small drug molecules, its failure to generate an acidic environment during degradation as compared to polylactides and glycolides, an exceptional ability to form blends with other polymers and slower degradation rates compared to PLGA [7]. PMMA is a biocompatible synthetic polymer with adequate mechanical strength for most of the biomedical and biotechnological applications [8].

In biomedical and pharmaceutical fields both nanoparticles and microparticles can be useful for different application. For example, nanoscale particles can be used to deliver drugs to target sites for cancer therapeutics or deliver imaging agents for cancer diagnostics because they travel through the blood stream without sedimentation or blockage of the microvasculature and can be taken up by cells through endocytosis at the target site. [3]. Although the definition identifies nanoparticles has having dimensions below 100 nm, in the biomedical field particles ranging between 100-500 nm may be also needed for loading a sufficient amount of active principle or signal inside. Microspheres in the range of 5-30 μm can be used as support matrices or drug delivery devices for site specific injection. Particularly, the size has to be tailored for a specific application that will affect a range of processes such as cell growth, tissue regeneration, drug release and host response [4].

In spite of the numerous advantages that biodegradable polymer particulates can provide, the development of such systems often poses a serious challenge due to lack of robust manufacturing techniques. Not only should these manufacturing techniques provide particles having predictable and controllable physical properties such as size distribution, composition, and structure; but, they must also conform to the rigorous requirements of product consistency, purity, and process scalability for pharmaceutical and biomedical applications [9].

Different conventional methods have been reported in literature for the preparation of micro and nanoparticles including, dispersion polymerization method [10], nanoprecipitation [11], solvent evaporation [12]. All these technology requires a final step of solvent extraction which involves expensive downstream processes to recover the solvents used.

Some supercritical fluid technologies have been proposed in the literature to produce biopolymeric microspheres [13]. A different approach, starting from emulsions and using SC-CO_2 for the extraction of the oily phase of the emulsion was proposed by different authors.
Particularly, SC-CO_2 has been proposed as extracting agent of the ‘‘oily’’ phase of oil-in water (o-w) emulsions to lead to solvent-free microparticles. The process, named supercritical emulsion extraction (or SEE), produces an aqueous suspension of microparticles after the supercritical extraction of the organic solvent contained in the emulsion micelles. In the SEE technology, the dimensions of the desired polymeric particles are directly connected to the dimensions of the droplets in the emulsions, indeed changing the emulsion formulation parameters it is possible to vary the dimensions of the particles produced after the solvent removal. The SEE process is a very fast process, due to the enhanced mass transfer of SC-

CO_2, capable of affecting also the size distribution of the produced microparticles since the fast extraction rate results in a narrower particle size distribution (PSD) because the droplets aggregation is minimized [14].

Recently, the emulsion extraction by SC-CO_2 was performed by using a process layout operating in continuous (SEE-C) by means of high-pressure packed tower for emulsion/SC-CO_2 contact in counter-current mode. The new proposed layout can overcome several disadvantages of the conventional solvent evaporation/extraction methods and produce micro and submicrospheres of different size and distribution in a robust and reproducible mode. The continuous process enhances the mass transfer due to a large contact area between SC-CO_2 and emulsions in the tower, allowing the production of microspheres more uniform in short processing times and a higher throughput with smaller plant volumes eliminating the batch-to-batch repeatability problems [15].

In our previous work, PLGA microspheres containing retinyl acetate were prepared by SEE-C using an innovative emulsion composition with non-halogenated solvents. The new emulsion is formed thanks to the phase separation between acetone (the solvent for the oily phase) and aqueous glycerol [16]. Particles in the micrometric range of dimensions have been successfully produced by SEE-C technology starting from this new emulsion formulation, with high encapsulation efficiency and very low solvent residue.

The aim of this work is to try to establish the conditions at which is possible to produce by SEE-C nanoparticles using acetone as disperse solvent of the emulsions. This work focuses on particle engineering from microparticles to nanoparticles for different common used biopolymers, obtained changing the formulation of the emulsion. Varying the emulsion formulation parameters such as, surfactant and biopolymer amount, phases ratio and emulsification technique (ultrasound or high speed emulsification), droplets dimensions have been changed from the microsize (mean size $\geq 0.5\mu m$) to the nanosize range (mean size < 400 nm), allowing the production of micro and nanoparticles of PMMA (poly-metylmetacrylate) and PCL (poly-caprolactone) of desired size and distribution after supercritical solvent extraction.

MATERIALS AND METHODS

Materials

CO_2 (99.9%, SON), polyvinyl alcohol (PVA, MW: 30.000–55.000, Aldrich Chemical Co.), acetone (A, purity 99.9%, Aldrich Chemical Co.), glycerol (purity 99%, Aldrich Chemical Co.), poly-caprolactone (PCL, MW: 14.000, Aldrich Chemical Co.), poly-metylmetacrylate (PMMA, MW: 20.000, Aldrich Chemical Co.) were used as received.

Apparatuse and Methods

For the O/W emulsions preparation, a known amount of polymer (from 1 to 10% w/w) was dissolved into 20 gr of acetone to form an organic solution. Then, the solution was added into a 80 gr of aqueous glycerol (20/80 w/w) PVA solution to form an emulsion using a high-speed stirrer and in some cases the emulsion obtained in this way was then sonicated to reduce the size of the formed droples (Digital Sonifier Branson mod. 45).

Supercritical emulsion extraction-continuous operation layout (SEE-C) apparatus consisted of a 107 cm long column with an internal diameter of 1.3 cm. The three stages are formed by stainless steel cylindrical elements of 30 cm height connected by four way (cross) unions and packed with stainless steel packings (1889m−1 specific surface; 0.94 of voidage; Pro- Pak). Cross-unions were also used to insert temperature controls at different heights of the column; the apparatus was thermally insulated by ceramic cloths and its temperature profile was controlled by six temperature controllers. SC-CO_2 was fed at the bottom of the column by a high-pressure diaphragm pump (model Milroyal B; Milton Roy) at a constant flow rate of 1.4 kg/h. The emulsion was taken from a reservoir and fed to the column by a high pressure piston pump (model 305; Gilson) at the top of the column at a constant flow rate of 0.15 kg/h (2.5 mL/min). A separator, located downstream the top of the column, was used to recover the extracted oily phase. The operation conditions were always 80 bar, 38°C and liquid to gas ratio 0.1, as previously optimized [16] with respect to the acetone residue. Particles suspension was continuously collected at the bottom of the column by decompression using a needle valve. At the end of each run, the suspension was washed several times by centrifugation at 6500 rpm for 45 minutes with distilled water, recovered by membrane filtration (0.1 µm porosity), and dried at air.

Conventional liquid emulsion extraction (LEE) of the oily solvent was also conducted for comparison purpose. Tipically, 20 g of emulsion was charged in a beaker and the solvent was extracted adding dropwise 60 g of aqueous-glycerol (ratio 50:50 water/glycerol), the resulting solution was stirred for 80 min at 400 rpm; subsequently, 40 g of pure water were added dropwise and the extraction was performed at the same stirring conditions for other 100 min.

Particles size distributions (PSD) of the recovered suspensions, were measured by dynamic light scattering with Malver zeta sizer instrument. A Field Emission-Scanning Electron Microscope (FE-SEM mod. LEO 1525) was used to study the morphology of the produced microspheres. A sample of powder was dispersed on a carbon tab previously stuck to an aluminum stub. Samples were coated with gold (layer thickness 250Å) using a sputter coater (mod.108 A, Agar Scientific).

RESULTS & DISCUSSION

Emulsion stability

It is known from the literature that when acetone, water, and glycerol are mixed, two phases can be formed due to phase separation in an appropriate (and relatively narrow) composition range. To obtain a stable emulsion, the ternary mixture composition should lie in this region. In our previous work [16] we verify that the optimal emulsion composition is O/W 20/80 with the water phase composed of a solution of aqueous glycerol (20% distilled water and 80% glycerol, by wait). For this reason in this work only the O/W 20/80 emulsion was used.

Supercritical Emulsion Extraction (SEE) sv Liquid Emulsion Extraction (LEE)

In this study, the comparison of the extraction processes (liquid and supercritical) is proposed based on the characteristics of the microspheres obtained by SEE-C and by conventional liquid extraction (LEE). The same emulsions (*o-w* ratio 20:80) have been processed with the two technologies.

In all cases studied, the suspensions produced by the supercritical fluids technology have smaller means size and narrower particles size distributions always located in the submicronic region, while the suspensions produced by LEE process are always characterized by wide particles size distribution that terminated over 1 µm. The PSD data of two different suspensions produced by SEE and LEE are reported for example in **Table 1**. The conventional liquid extraction suffers of coalescence and aggregation problems typically due to an inadequate amount of the extraction agent or an insufficient absorbing rate of the solvent leached from the solidifying microspheres, that may generate intra-particle adhesion. This phenomena put a limit to this technology that is, for this reason, not suitable for nanoparticles production.

	PCL5%		**PMMA5%**	
	SEE	*LEE*	*SEE*	*LEE*
MD(nm)	342	710	455	1075
PDI	0.19	0.12	0.21	0.22

Table 1. Means Diameters (MD) and Polydispersity Index (PDI) for different suspensions produced by Supercritical Extraction of Emulsion (SEE) and Liquid Extraction of Emulsion (LEE). The suspensions reported in this table are obtained from emulsions: O/W 20/80, PVA 1%, emulsified at 7000 rpm for 6 min, and sonicated for 1 min at 30%.

The particles size distribution of the obtained suspensions was also plotted together for comparison in **Figure 1**, where it is evident the enlargement of the PSD in the case of LEE produced suspensions.

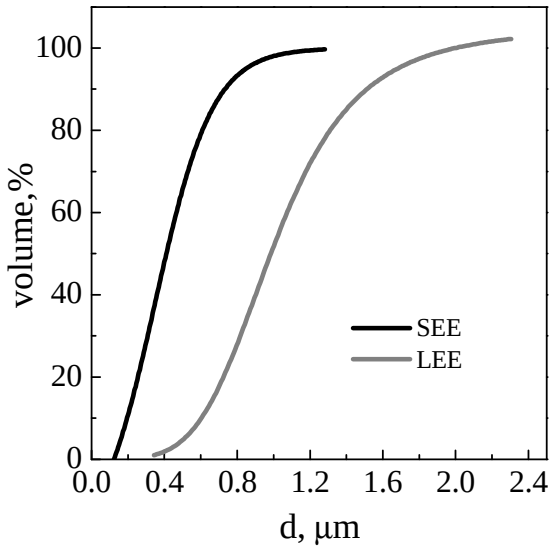

Figure 1. Cumulative nanosizer particles size distribution of the suspensions obtained by SEE and LEE technology for PCL5% experiment.

The morphology of microspheres produced by SEE-C was in all cases spherical and no coalescing; however, collapsed particles were often produced by LEE.

Particles engineering by SEE: from micro to nanoparticles

Several parameters affecting the final droplets and then particles dimensions where studied (concentration of surfactant, biopolymer concentration, ratio between the phases of the emulsion, ultrasound emulsification and high speed emulsification conditions). In the following the most effective parameters, such us polymer concentration in the oil phase and surfactant concentration in the water phase, are reported. The effect of emulsification conditions was also studied and emulsification parameters were optimized. All the suspensions reported in the following were obtained stating from emulsions produced at the optimized condition of emulsification: high speed emulsification (7000 rpm for 6 minutes) and then ultrasound emulsification (amplitude 30% for 1 minute).

The effect of PCL and PMMA concentration in the oily phase was tested on particles size distribution. For these purposes, the composition of the water phase was maintained constant (80% glycerol, 20% water and 1% PVA), varying only the composition of the oil phase. The ratio between oil and water was also fixed (O/W:20/80), taking into account the phase separation boundary into the ternary phase diagram, previously discussed. A summary of the results obtained for the two polymers is reported in **Table 2**.

	MD (nm)	PDI
PCL1%	342	0.16
PCL5%	342	0.19
PCL10%	761	0.25
PMMA1%	455	0.21
PMMA5%	571	0.4

Table 2. Effect of PCL and PMMA concentration in oily phase. Means Diameters (MD) and Polydispersity Index (PDI) for different suspensions produced by Supercritical Extraction of Emulsion (SEE)

Increasing the polymer content in the oily phase of the emulsion there is an evident increase of the mean diameters of the particles and of the polydispersity index. In the case of PCL system changing the polymer concentration from 1 to 10% it is possible to tune the particles size dimensions from 342±54 to 761±190, as can be seen in **Figure 2**. In the case of PMMA system nanoparticles with 455±95 are obtained with 1% of polymer in oil phase and microparticles with 571±228 with 5% of polymer concentration.

From **Figure 2** and **Table 2**, it is also evident that the smallest nanoparticles with PDI very close to 0.1 (monodisperse particles suspensions) are obtained with PCL, and the particles obtained with PMMA are always larger then that produced with PCL in the same conditions and also with greater dispersion of the dimensions

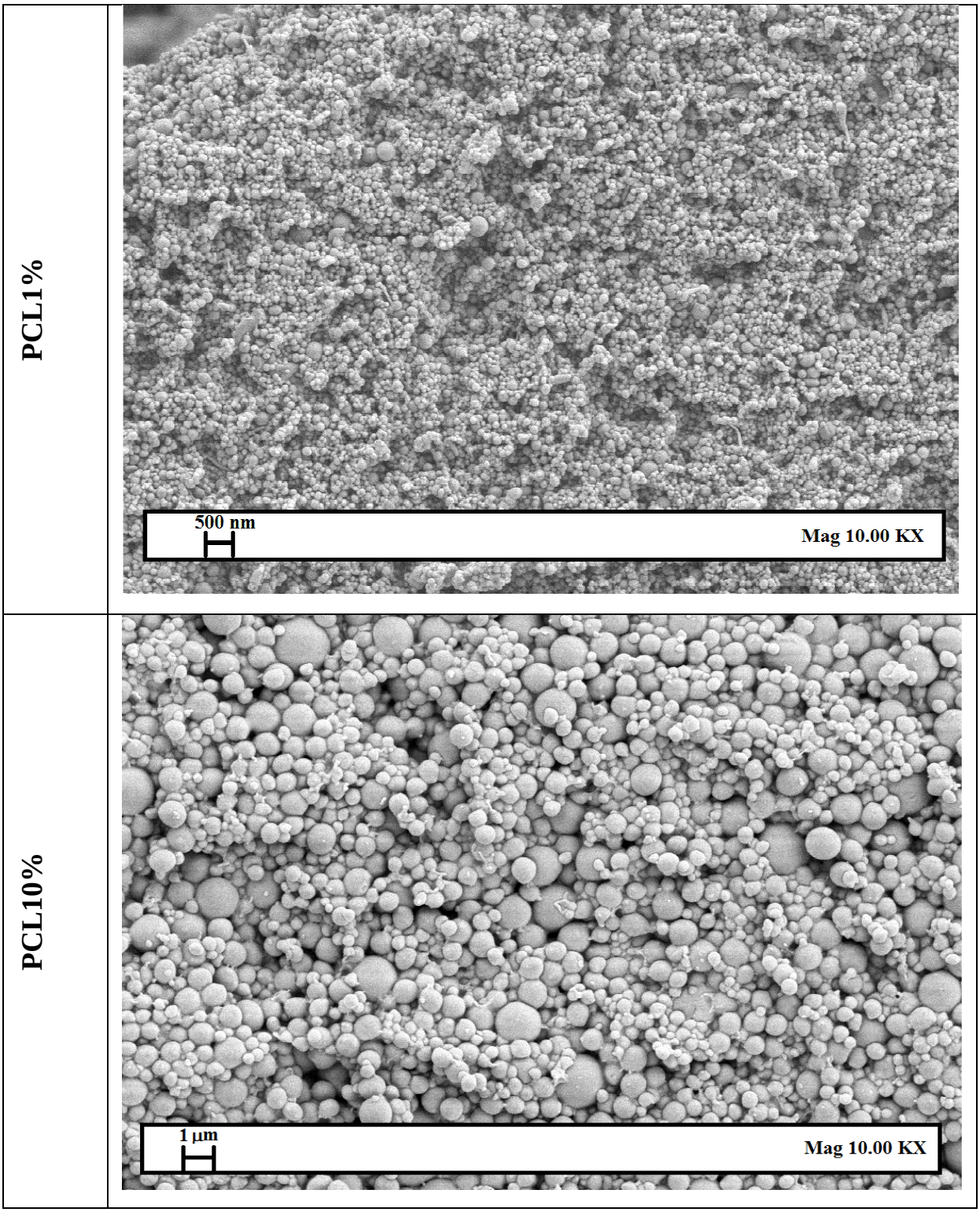

Figure2. SEM images of micro and nanoparticles of PCL

The effect of the concentration of the surfactant was also investigated. As reported in **Figure 3** and in **Figure 4**, the PVA concentration in the water phase of the emulsion have been varied from 0.6% ,1% and 2%, fixing the O/W ratio to 20/80 and 1% w/w PCL concentration in the oil phase. The effect of the surfactant is of great importance in this case, because increasing its concentration it allows to change the dimensions of the droplets from the micrometric region to the submicrometric one, and conseguently the dimensions of the particles after the supercritical solvent extraction.

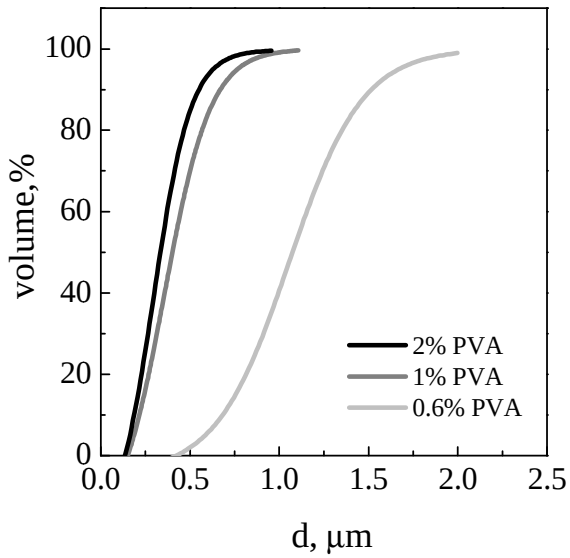

Figure 3. Cumulative particle size distribution of the PCL suspensions obtained with different PVA concentration

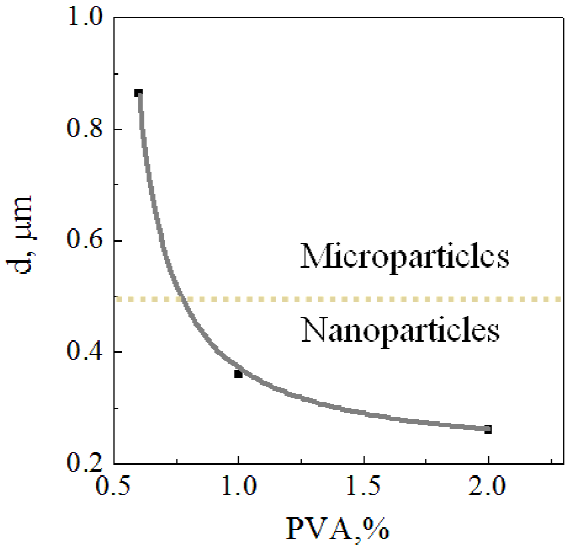

Figure 4. Mean Diameters of PCL nanoparticles obtained with different concentration of PVA as surfactant of the water phase

The PVA concentration was further increased in the attempt to obtain particles with smaller dimensions, but increasing the concentration of the surfactant (3% w/w and 4% w/w) the consequent increase of the viscosity of the external phase prevent the reduction of the droplet, producing analogous results of the 1% PVA experiment.

CONCLSIONS

The SEE technology was successfully applied for the production of micro and nanospheres. The supercritical process allows the production of smaller particles with respect to the conventional one. Indeed the conventional process fails in the production of nanoparticles in consequence of high aggregation phenomena. While, the SEE process exhibit high flexibility, allowing to tune the dimension of the particles easily changing the formulation of the starting emulsion. Thanks to the enhanced mass transfer of the supercritical fluids and the more efficient solvent removal stable suspensions of micro and nanoparticles were always obtained.

REFERENCES

[1] LAKSHMI, S., CATO, T., Adv Biochem Engin/Biotechnol Vol. 102, **200**6, p. 47
[2] FREIBERG, S., ZHU, XX., Int J Pharm Vol. 282, **2004**, p 1
[3] BRANNON-PEPPAS, L., BLANCHETTE, J.O., Advanced drug delivery reviews **2004**
[4] JAKLENEC, A., HINCKFUSS, A., BILGEN, B.,CIOMBOR, D., AARON, R., MATHIOWITZ, E., Biomaterials, Vol. 29, **2008**, p. 1518
[5] LASSALLE, V., FERREIRA, M., Macromol. Biosci. Vol 7, **2007**, p. 767
[6] BENNY, O., MENON, L. G., ARIEL, G., GOREN, E., KIM, S., STEWMAN, C., BLACK, P.M., CARROLL, R.S., MACHLUF, M., Clinical. Cancer Research, Vol. 15, **2009**, p. 1222
[7] SINHA, V.R., BANSAL, K., KAUSHIK, R., KUMRIA, R., Trehan, A., International Journal of Pharmaceutics, Vol. 278, **2004**, p. 1
[8] SONGJUN, L., JIE, H., BAILING, L., BioSystems, Vol 77, **2004** , p. 25
[9] CHATTOPADHYAY, P., HUFFB SHEKUNOV, R.,. J of Pharm Sci, Vol 95, **2006,** p.667
[10] ZHANG, X., SHEN, S., FAN, L., Polymer Bulletin, Vol. 61, **2008**, p.19
[11] BARICHELLO, J.M.;,MORISHITA, M., TAKAYAMA, K., NAGAI, T., Drug Development and Industrial Pharmacy , Vol. 25, **1999**, p. 471
[12] FENG, S., HUANG, G., Journal of Controlled Release, Vol. 71, **2001**, p. 53
[13] REVERCHON, E., ADAMI, R., CARDEA, S., DELLA PORTA, G., J Supercrit Fluids, Vol. 47, **2009**, p. 484
[14] DELLA PORTA, G., REVERCHON, E., Biotechnol Bioeng, Vol. 100, **2008**, p.1020
[15] DELLA PORTA, G., FALCO, N., REVERCHON, E., Biotechnol Bioeng, Vol. 108, **2011**, p. 676
[16] DELLA PORTA, G., CAMPARDELLI, R., FALCO, N., REVERCHON, E., J Pharm Sci, **2011,** DOI 10.1002/jps.