CONTINUOUS SUPERCRITICAL FLUID EMULSIONS EXTRACTION: CAPABILITIES AND PERFORMANCES OF AN INNOVATIVE PROCESS FOR BIOPOLYMER MICROSPHERES PRODUCTION

Della Porta G. [(a-b)], **Falco N.** * [(a)], **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: nfalco@unisa.it

ABSTRACT

The aim of the present work is to demonstrate the capabilities of the continuous supercritical emulsion extraction technology (SEE-C) for the production of *poly-lactic-co-glycolic acid* (PLGA) microspheres with an engineered size and distribution charged with different active principles. Indeed, varying the emulsion formulation and the droplet dimensions, microspheres charged with different active principles and with different mean diameters can be produced in few minutes by SEE-C operating in a pressure range of 80-120 bar at 38°C with a liquid/gas flow-rate ratio of 1/10. For all the systems studied, a detailed characterization of the produced microspheres in terms of morphology and size distribution is proposed together with a study of the release profiles of the entrapped active principles.

INTRODUCTION

Several products based on active principle-loaded biodegradable microspheres have reached the pharmaceutical market place. Injectable depot formulations can provide sustained and controlled delivery of the active over a period of weeks or months and thus significantly increase patients' comfort and compliance. Although microsphere-based active delivery is attractive from both the market and patient perspective, developers of microsphere formulations face many challenges in achieving the desired product performance and process efficiency. An important parameter for robust active formulation is the microsphere size. Indeed, many of the challenges in achieving the desired product performance and process efficiency are related to the lack of control over particle size and uniformity of conventional microsphere manufacturing methods. There are a number of techniques in development designed to overcome issues regarding size and uniformity [1]. A novel manufacturing process called Supercritical Emulsion Extraction (SEE) was proposed for the production of active principle/polymer microspheres with controlled particle size distribution, starting from single *oil-in-water (o-w)* and double *water-oil-water (w-o-w)* emulsions. Particles formation in emulsion is achieved by removal of the internal organic oil phase from the emulsion

droplets by extraction using SC-CO_2 [2-3]. This technology was first proposed in batch configuration. The major limitation of SEE batch process layout, shared with traditional evaporation processes, is the intrinsically discontinuous operation such as problems of batch-to-batch variability and reduction of the process yield [4-5]. Recently, the Supercritical Emulsion Extraction process was described in a Continuous operating mode (SEE-C) as much faster and selective than the conventional solvent evaporation/extraction due to the enhanced mass transfer, and capable of the production of micro and sub-microspheres with different size and distribution in a robust and reproducible mode. The innovative process arrangement is obtained by using a high-pressure packed tower operating in countercurrent, in which mass transfer between the liquid and the gaseous phase is improved by the internal packing elements [6].

The aim of the present work is to demonstrate the capabilities and the performances of the continuous SEE-C process for the production of poly-lactic-co-glycolic acid (PLGA) microparticles with an engineered size and distribution and charged with different active principles. Poly-lactic-co-glycolic acid (PLGA) is a well-know biodegradable and biocompatible polymer used for controlled release and FDA approved for injectable devices production. The extraction experiments have been performed using both single *o-w* and *w-o-w* emulsions; different PLGA/active principle systems were tested. Particularly, anti-inflammatories drugs (such as Piroxicam and Diclofenac Sodium), corticosteroids (such as Hydrocortisone acetate) and peptides and proteins (such as Samyr and Insulin) were selected as active principles to be entrapped. For all the systems studied, a detailed characterization of the produced microspheres in terms of morphology and size distribution is proposed on the different produced devices. Active principles release profiles of the microspheres produced are also proposed to obtain further information about the particles structure.

EXPERIMENTAL METHODS

Materials and Methods

CO$_2$ (99.9%, SON, Italy), polyvinyl alcohol (PVA, Mol wt: 30000–55000, Aldrich Chemical Co. Italy), ethyl acetate (EA, 99.9%, Aldrich Chemical Co., Italy), poly-(lactic/glycolic) acid (PLGA 50:50, RESOMER® RG 504H, Mol wt: 38000-54000, Boehringer Ingelheim, Germany; PLGA 75:25, Mol wt: 60000-120000 Aldrich Chemical Co., Italy), Piroxicam (PX, purity 99.9%, Sigma-Aldrich Co., Milan, Italy), Diclofenac Sodium (DS, purity 99.9%, Sigma-Aldrich Co., Italy), Samyr (SAM, purity 99.9%, Abbott, Italy), Hydrocortisone Acetate (HA, purity 99.9%, Sigma-Aldrich Co., Italy), Insulin (INS, purity 99.9%, Sigma-Aldrich Co., Italy) were used as received.

Emulsion preparation

Single *o-w* emulsions (composition ratio: 20:80 w/w) were prepared dissolving a fixed amount of active principle-polymer mixture into EA and then, emulsifying it with water/PVA solutions (0.8% w/w of PVA) using a high-speed stirrer (mod. L4RT, Silverson Machines Ltd., Waterside, Chesham Bucks, United Kingdom) operating at 2.800 rpm for 3 min.
Double *w-o-w emulsions* (composition ratio: 1:19:80 w/w/w or 2:18:80, depending on the test performed) were prepared pre-suspending a know amount of active principle in 1-2 mL of water/PVA solution (in the case of Diclofenac Sodium, Samyr and Insulin) or *n*-ethanol/PVA (in the case of Hydrocortisone acetate) (0.04-0.06% w/w of PVA), that was added into polymer/EA fixed solutions and sonicated (mod. VCX130-Vibra Cell, Sonics and Materials, Newtown, Connecticut, USA) at 50% of amplitude for 2 min. The primary *w-o emulsion* was,

then, added into water/PVA solutions (0.6-0.8% w/w in water) to form the secondary emulsion using a high-speed stirrer operated at 2800 rpm for 3 min (in the case of Insulin) or 6 min (in the case of Diclofenac Sodium, Samyr and Hydrocortisone acetate). The extraction experiments have been performed varying the polymer concentration from 5% to 10% w/w, depending on the test performed.

SEE-CM apparatus

The SEE-C process scheme is reported in **Figure 1**. Process equipment consisted of a packed column where gaseous and liquid phases (SC-CO_2 and emulsion) were contacted counter currently. The column consisted of three extraction stages formed by AISI 316 stainless steel cylindrical sections of 30 cm height, connected by four cross-unions. The column was packed with stainless steel packings (1889 m^{-1} specific surface and 0.94 of voidage; Pro-Pak, Scientific Development Company, State College, Pennsylvania, USA) and thermally insulated by ceramic cloths. Temperature profile was controlled by six controllers inserted at different heights of the column by the cross-unions. A separator located downstream the column was used for recovering the extracted solvent. A high-pressure diaphragm pump (mod. Milroyal B, Milton Roy, Pont Saint-Pierre, France) was used for CO_2 delivering at a constant flow rate of 1.4 Kg/h and a high pressure piston pump (mod. 305, Gilson, France) was used for feeding the emulsion at a constant flow rate of 2.5 mL/min. A rotameter and a dry test meter located at the exit of the separator measure the CO_2 flow rate and the total quantity of CO_2 delivered, respectively. The emulsion was introduced at the top of the column and the CO_2 was fed from the bottom of the column. Microspheres suspension was continuously collected at the bottom of the extraction column. Then, microspheres were washed by centrifugation with distilled water, recovered by membrane filtration and dried at air for further processing. At the end of each run, the column was washed with distilled water to eliminate any processing residue from the packing surface.

Droplets and microspheres morphology and size distribution

The droplets contained in the emulsions were observed using an optical microscope (mod. BX 50 Olympus, Tokyo, Japan) equipped with a phase contrast condenser. The morphology of the produced microspheres was observed using a Field Emission-Scanning Electron Microscope (FE-SEM mod. LEO 1525, Carl Zeiss SMT AG, Oberkochen, Germany). Droplet size distributions (DSDs) and particle size distributions (PSDs) were measured by dynamic light scattering (DLS, mod. Mastersizer S, Malvern Instruments Ltd., Worcherstershire, United Kingdom). Analyses were performed immediately after the preparation of the emulsion and of the microsphere suspension, using several milligrams of each sample (corresponding to more than one million of droplets or particles). DLS tests were repeated ten times for each sample. Mean droplet/particle size and polidispersity index were calculated for each sample.

Solvent Residue Analysis

The EA content in the suspensions was analyzed to check the efficiency of solvent removal from the emulsion. The EA residue was measured using a head space sampler (mod. 50 Scan; Hewlett–Packard, Palo Alto, California) coupled to a gas chromatograph interfaced with a flame ionization detector (GC–FID, mod. 6890 Agilent Series; Agilent Technologies Inc., Wilmington, Delaware). EA was separated using a silica capillary column 30 m length, 0.25 mm internal diameter, 0.25 µm film thickness (mod. DB-1; J&W, Folsom, California). The oven temperature in the GC was set to 40°C for 8 min. The injector was maintained at

180°C (split mode, ratio 1:1) and Helium was used as the carrier gas (7 mL/min). Head space conditions were: equilibration time 60 min at 100 °C, pressurization time 2 min, loop fill time 1 min. Head space samples were prepared in 10 mL vials filled with 3 mL of suspension. Analyses were performed on each sample in three replicates.
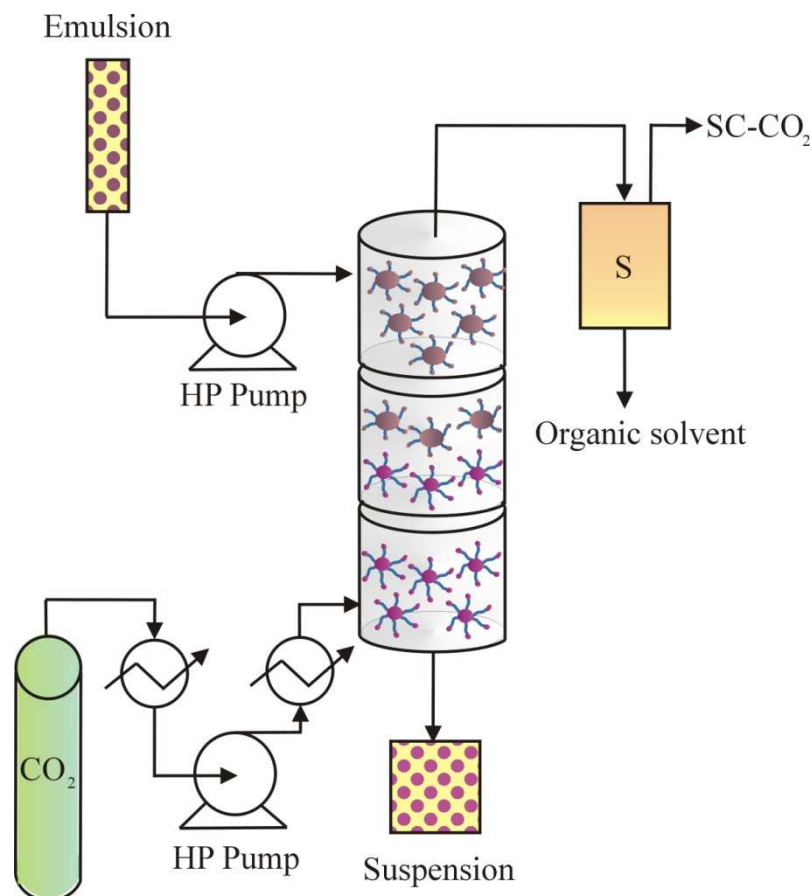

Figure 1: Schematic representation of the Supercritical Emulsion Extraction operating in Continuous mode (SEE-C) apparatus layout.

Active principle loading and release

Active principle loading refers to the amount of recovered active principle in the microspheres with respect to the amount initially used. Active principle loading was determined by dissolving a known mass (2.5-10 mg) of microspheres in 1 mL of a given solvent. Samples were stirred for minutes or hours at 100 rpm to ensure the complete dissolution of the polymer. Pure active principles were also dissolved to verify their stability in the solvent and no degradation was observed. The concentration of active principle in the resulting solution was determined by HPLC (model 1200 series, Agilent Technologies Inc., Italy) or by an UV–Vis spectrophotometer (mod. Cary 50, Varian, Palo Alto, California).

PX, DS, HA release rates were determined using the UV–Vis spectrophotometer. Aliquots (100 mg) of drug-loaded microspheres were pre-suspended in 2 mL of release medium and charged into a dialysis sack. Drug release profiles were determined in 200 mL of phosphate-buffered saline (PBS 0.2 M, pH 7.4) continuously stirred at 100 rpm in a 37°C incubator to maintain adequate sink conditions. SAM and INS release profiles were

monitored by HPLC. Similarly, dissolution profiles of the reference samples of pure active principle were also monitored for comparison purpose. Each analysis was performed in triplicate and the proposed curves are the mean profiles obtained.

RESULTS AND DISCUSSION

SEE-C Process Parameters

In SEE-C processes, processing pressure and temperature conditions were selected to enhance the extraction of the oily dispersed phase of the emulsion, but also to avoid active principle or polymer losses by dissolution in $SC-CO_2$ and to avoid the emulsion loss by washing out in the $SC-CO_2$ stream. Optimized operating conditions were found to be in a pressure range of 80-120 bar at 38°C, according to the high pressure phase diagram of the system $EA/SC-CO_2$ [7-9]. The liquid and the gas come in contact inside the column by means of the packing material; in this case, good separations were obtained fixing the ratio between the emulsion and $SC-CO_2$ (L/G) at 0.1. Particularly, the $SC-CO_2$ and liquid flow rates used were 1.4 kg/h and 0.14 kg/h (2.3 mL/min of emulsion), respectively [6;10]. As a consequence, in this work, all the emulsions were processed using these process conditions.

Examples of Droplets and Microspheres Produced

Single and double emulsions were produced varying the PLGA % in the oily phase to obtain droplets (i.e., particles) of different sizes. Different active principle/PLGA systems were tested. A single *o-w* emulsion was used to encapsulate PX in PLGA, due to the good solubility of both PX and PLGA in EA. A double *w-o-w* emulsion was used to encapsulate DF, HA, SAM and INS in PLGA, due to their insolubility in EA. In the case of double emulsions, more correctly microcapsules are obtained than microspheres, since a shell of polymer covers the microparticle. Double emulsions are more difficult to be processed since the emulsion can be less stable and the particles can be porous; therefore, coalescence phenomena can happen and a large part of the active principle can be lost in the aqueous phase. DSDs and related PSDs parameters of the processed emulsions are reported in details in **Tables 1.** In the same table, the experimental active principle loading, the solvent residue value of all discussed microspheres and the polydispersity index (CV, defined as the ratio between the standard deviation and the mean size of the same distribution) are also summarized. PLGA amount in the oily phase was varied from 5% w/w (in the case of PX, DF and HA) to 10% w/w (in the case of SAM and INS). Droplets size largely depends on the PLGA content in the oily phase: an increase of PLGA percentage produced larger droplets and the formation of wider DSDs, when using the same dispersion shear force and surfactant concentration; whereas, droplet size is relatively not dependent on the kind of active principle and emulsion (single or double). The presence of PLGA 5% w/w produced droplets ranging approximately between 1.0 µm and 3.5 µm; in the case of PLGA 10% w/w the distribution ranged between 1.5 µm and 9.0 µm. Particularly, mean droplet sizes of 2.1 µm (SD 0.9) and 4.7 µm (SD 2.4) were obtained with PLGA amounts of 5% and 10% w/w, respectively. The experimental evidence is that the SEE-C technology produced always microspheres with smaller mean sizes and narrower PSDs than the droplet mean sizes and the DSDs of the starting emulsion, due to a very short processing time. As expected, the increase of the emulsion droplets size with PLGA concentration produced a significant increase in PSDs of the obtained microspheres. Microspheres obtained from emulsions containing 5% w/w of PLGA cover approximately a range between 0.5 and 2.5 µm; microspheres produced from emulsions containing 10% w/w of PLGA cover a range between 1.0 and 7.5 µm. The mean
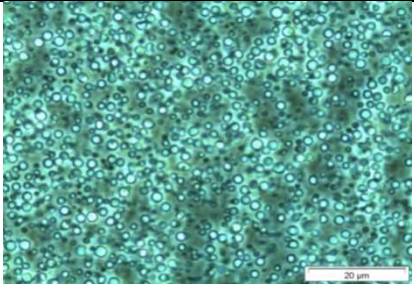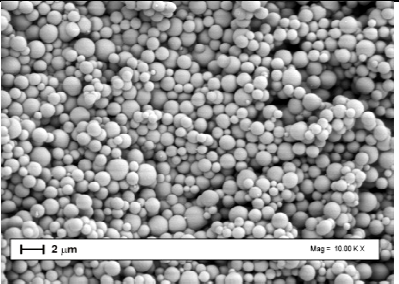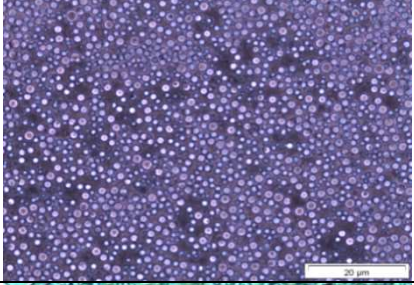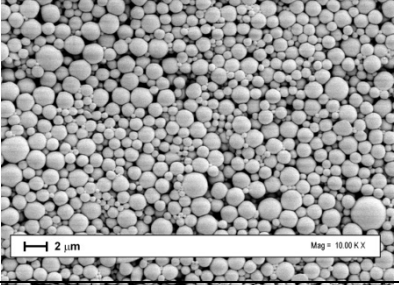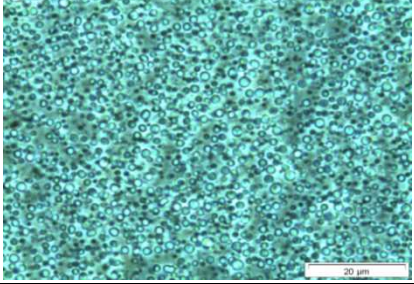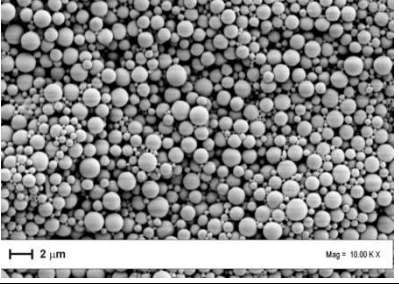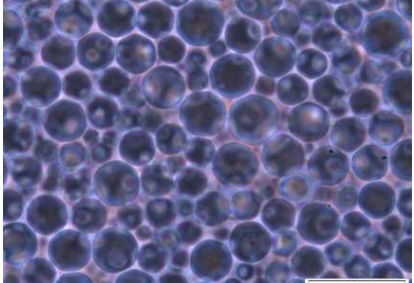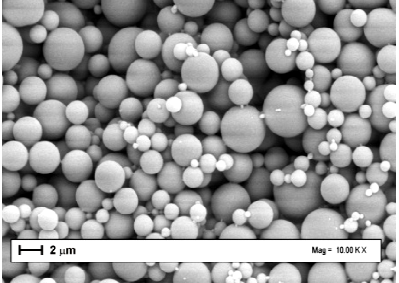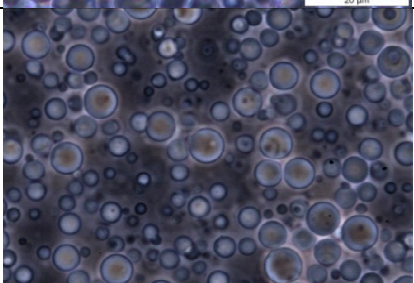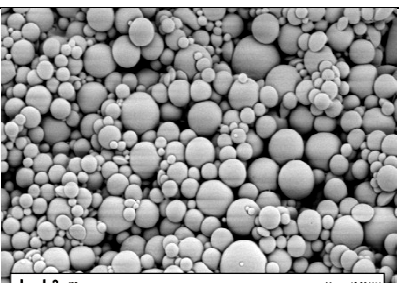
particle size varied from 1.7 µm (SD ±0.8) to 4.1 µm (SD ±2.1) when the PLGA concentration was varied from 5% to 10% w/w, respectively. The CV of the microspheres containing PLGA in concentration of 5% w/w had always a higher values of the CV of the corresponding emulsion; whereas, the CV of the microspheres containing 10% w/w of PLGA had a smaller value with respect to the one of the related emulsion. Shrinking factor values (SF, defined as the ratio between the mean size of the microspheres in suspension and the mean size of the corresponding droplets in the emulsion) were measured in the range 0.5-0.9, indicating that the particles produced are almost always smaller than the original droplets. These results confirm the efficiency of the continuous SEE-C process, since all the phenomena of growing of the particles and their coalescence that can affect the solvent evaporation process have been avoided. The reasons of the success of this process are in the very short processing time of the emulsion. The solvent residue value (also reported in **Table 1**) ranged between 300 and 1000 ppm. This value is considerable lower than the ones (between 2000-3000 ppm) measured in the conventional solvent evaporation process. Indeed, the continuous process enhances the mass transfer due to a better contact between emulsion and SC-CO_2 and, therefore, best performances are obtained. The continuous processing also allowed encapsulation efficiencies between 30-90% and the recovery of maximum 85% of the charged material.

Table 1: Size distribution data of microspheres produced by SEE-C using *o-w* and *w-o-w* emulsions. Encapsulation efficiency and solvent residue values are also reported. Legend: MS = mean size; SD = standard deviation; CV = coefficient of variation.

Active principle	PX	DS	HA	SA	INS
PLGA composition (PGA/PLA)	75:25	75:25	50:50	75:25	50:50
Emulsion	*o-w*	*w-o-w*	*w-o-w*	*w-o-w*	*w-o-w*
% Active principle charged **% PLGA**	14 5	9 5	1.4 5	3 10	0.35 10
MS [µm]	1.7	1.9	1.8	4.1	3.1
SD [µm]	0.8	0.8	0.8	2.1	1.5
CV [%]	47	42	44	51	48
D_{10} [µm]	0.6	0.7	0.7	1.3	0.9
D_{50} [µm]	1.0	1.3	1.1	2.7	1.8
D_{90} [µm]	1.6	2.3	1.9	4.7	3.3
Solvent residue [ppm]	533	644	938	711	340
Encapsulation efficiency (%)	91	85	50	30	63

The variation of PLGA concentration in the oil phase from 5% to 10% w/w produced emulsions with stable and non-coalescing droplets, as illustrated in the optical microscope image reported in **Figures 2a.** The correspondent microspheres obtained after SEE-C treatment of *o-w* and *w-o-w* emulsions prepared with PLGA concentrations of 5% and 10% w/w are reported in the FE-SEM images shown in **Figure 2b**. Very well-defined spherical, not agglomerated and uniform in size microspheres were produced in the range 80-120 bar at

38°C.

Encapsulated active principle	Emulsion type	Optical microscope images (a)	FE-SEM images (b)
Piroxicam	o-w		
Diclofenac Sodium	w-o-w		
Hydrortisone acetate	w-o-w		
Samyr	w-o-w		
Insulin	w-o-w		

Figures 2: (a) Optical Microscope (MO) images of *o-w* and *w-o-w* emulsions produced varying the PLGA concentration from 5% to 10% w/w; **(b)** FE-SEM images of the related microspheres produced by SEE-C process.

Anti-inflammatory, corticosteroid, protein release profiles

PX, HA and INS release tests were performed on microspheres with different mean sizes (see **Table 1**) to monitor the efficiency in active principle release of the microcomposite particles produced by SEE-C process. It is known from literature **[11]** that active principle release from PLGA can be described in two steps: diffusion through the polymer and bulk erosion of the polymer itself. These two mechanisms can overlap or operate in sequence, depending on the polymer molecular weight and mainly on the polymer composition, that is the ratio poly-lactic to poly-glycolic acid (PLA/PGA). PLGA microspheres with a mean size of 1.7 µm (SD ± 0.8 µm) and with PX effective loading of 14% w/w were tested for drug release studies in a release medium of phosphate buffered solution (PBS 0.2 M, pH 7.4) with 0.5% Tween-80 at 37°C. The release profile data are reported in **Figure 3a**; in the same figure the dissolution rate of pure PX is also reported, for comparison. Pure PX was dissolved in 350 min. A slower drug release was obtained from loaded microspheres: i.e., the 85% of PX was released in 8 days when 14% w/w of PX was charged. The release profile illustrated in **Figure 3a** also shows the evidence of two distinct aliquots: about the 50% of the drug seems available for diffusion; whereas, the remaining part seems to be blocked inside the polymer and cannot be reached by diffusion alone.

To test the HA release from the SEE-C produced devices, PLGA microspheres with a MS of about 1.8 mm (SD 0.8) and with HA loading of 1.4% w/w were studied. The results obtained are reported in **Figure 3b**; in the same figure the dissolution rate of pure HA is also reported, for comparison. Pure HA was dissolved in 160 min. The total drug release from microspheres was obtained in about 6 days when 1.4% w/w of the HA was charged. In this case all the charged drug was released prevalently by diffusion because PLGA with a PGA/PLA copolymer ratio of 50:50 was used. The overall drug release times measured for HA were about half of those measured for PX. This behavior maybe is due to the fact that probably HA is not blocked into the polymer forming the microspheres, since these microspheres are produced using a double emulsion instead of a single emulsion. Indeed, it is well know that microspheres produced starting from double emulsions can be porous due to the presence, during their formation, of a water internal phase that will induce the formation of partially empty structures with micropores distributed inside the microsphere **[12]**. In such porous PLGA structures, the diffusion of water from the external medium is expected to be facilitated and, consequently, the resulted diffusion times are shorter.
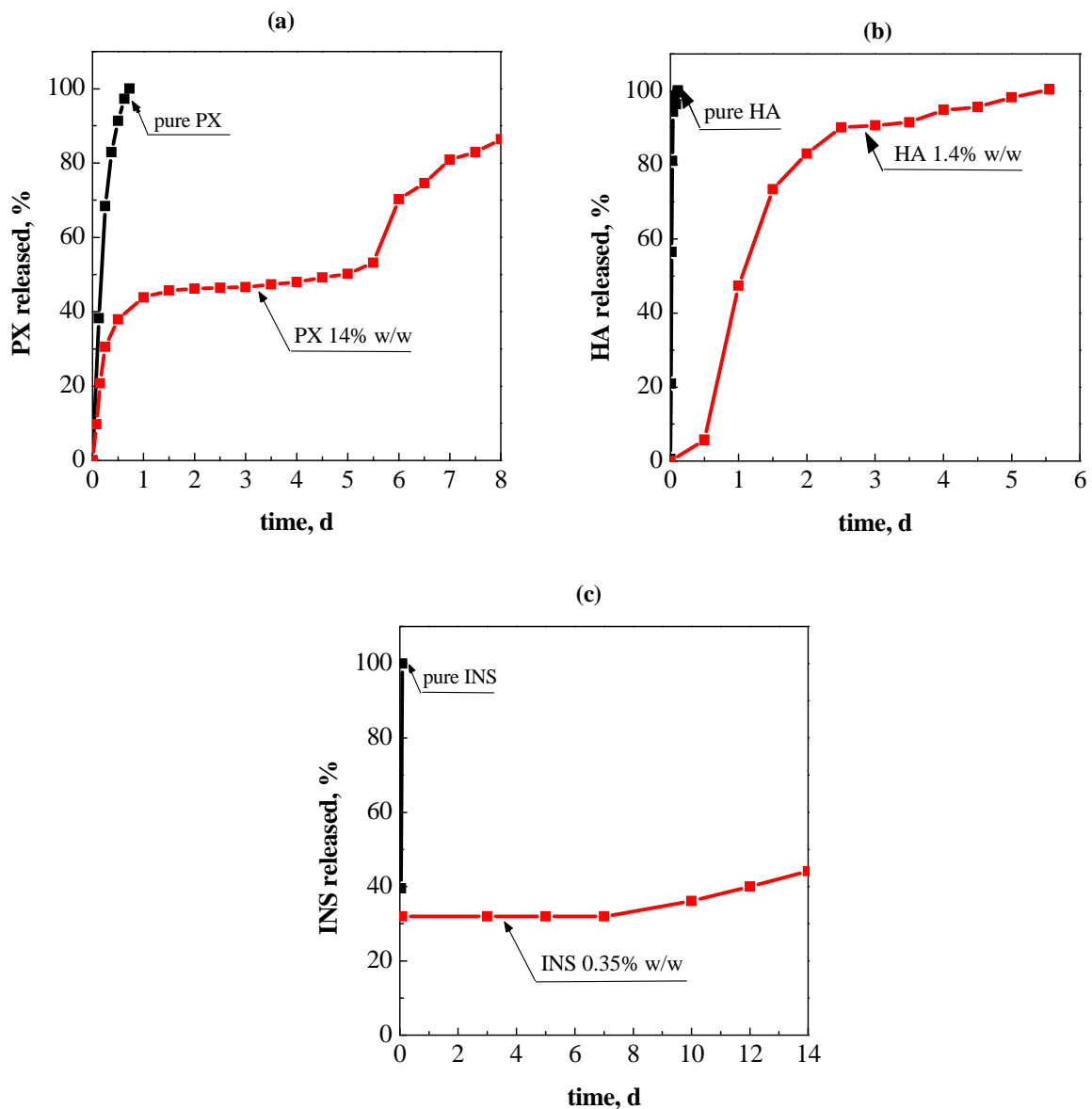
Figure 3a-c: In vitro release profiles from PLGA microspheres loaded with different active principles: **(a)** PX 15% w/w, **(b)** HA 1.4 % w/w and **(c)** INS 0.35% w/w. The release profile of pure active principles are also proposed in each diagram, for comparison.

The Insulin release profile is reported in **Figure 3c**; in the same figure the dissolution rate of pure Insulin is also reported, for comparison. Pure INS was completely dissolved in 2 hours. A slower drug release from microspheres is obtained: i.e., the 45% of the Insulin was released in 14 days when the active principle charged was the 0.35% w/w. In the first 2 hours the 32% of the encapsulated Insulin from microspheres. This high initial burst release of Insulin is due to the rapid diffusion of the Insulin molecules located on the surface. Further, since Insulin is a small protein, molecules located even at the core of the microspheres can diffuse out fast as soon as the pores are formed due to the water penetration after incubation. The high initial burst release was followed by a slow release and subsequent incomplete release. The incomplete release observed for Insulin can be due to the formation of covalent or non-covalent aggregates during microsphere fabrication and/or adsorption of proteins to the PLGA

matrix, as suggested by Manoharan et al. **[13]**. In general, interactions between PLGA and encapsulated proteins can be hydrophobic or electrostatic. It has been reported that Insulin adsorbs to some hydrophobic surfaces resulting in protein unfolding and aggregation. Interactions between PLGA and the encapsulated Insulin results in the formation water insoluble non-covalent aggregates. Thus they are not detected during protein content estimation and do not yield cumulative release data.

REFERENCES

[1] VELDHUIS, G., GIRONÈS, M., BINGHAM, D., Drug Delivery Technology, Vol. 9(1), **2009**.
[2] CHATTOPADHYAY, P., HUFF, R., SHEKUNOV, B.Y., J Pharm Sci, Vol. 95, **2006**, p. 667.
[3] DELLA PORTA, G., REVERCHON, E., Biotech Bioeng, Vol. 100(5), **2008**, p. 1020.
[4] DELLA PORTA, G., FALCO, N., REVERCHON, E., J Pharm Sci, Vol. 99(3), **2010**, p. 1484.
[5] KLUGE, J., FUSARO, F., CASAS, N., MAZZOTTI, M., MUHRER, G., J Supercrit Fluids, Vol. 50, **2009**, p. 327.
[6] DELLA PORTA, G., FALCO, N., REVERCHON, E., Biotech Bioeng Vol. 108(3), **2011**, p. 676.
[7] DELLA PORTA, G., VOLPE, M.C., REVERCHON, E., J Supercrit Fluids, Vol. 37, **2006**, p. 409.
[8] SMITH, R.L., YAMAGUCHI, T., SATO, T., SUZUKI, H., ARAI, K., J Supercrit Fluids, Vol. 13, **1998**, p. 29.
[9] KING, M.B., MUBARAK, A., KIM, J.D., BOTT, T.R., J Supercrit Fluids, Vol. 5, **1992**, p. 296.
[10] BRUNNER, G., J Supercrit Fluids, Vol. 47, **2009**, p. 574.
[11] SIEPMANN, J., GOPFERICH, A., Adv Drug Del Rev, Vol. 48, **2001**, p. 229.
[12] LI, M., ROUAND, O., PONCELET, D., Int J Pharm, Vol. 363, **2008**, p. 26.
[13] MANOHARAN, C., SINGH, J., J Pharm Sci, Vol. 98(2), **2009**, p. 529.