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Abstract

Electrospray has been recognized as an efficient technique for the fabrication of polymer micro and nanosystems and recently it was applied to lipids. The objective of this study was to assess the potential of electrospray for the encapsulation of insulin into lipid particles. Spherical particles of about 1 lm were obtained jetting a propanolic solution of palmitic or stearic acid and ethylcellulose or Pluronic F127 in a 10:1 or 20:1 (w/w) ratio under an electric field of 30 kV. Insulin was entrapped into the particles with high encapsulation efficiency by the formation of an ion-pair with sodium dodecyl sulphate. Far-ultraviolet circular dichroism spectroscopy indicated that electrospray did not modify the secondary structure of insulin. An in vitro prolonged release over 24 hours was observed after an initial burst effect. This study demonstrates that electrospray represents a viable new alternative for preparing in a single step peptide-protein loaded lipid based microspheres directly in powder form.

Keywords: Drug delivery systems, electrospray, insulin, solid lipid microparticles

INTRODUCTION

Nowadays, solid lipid micro- and nanoparticles represent a new form of particulate carriers for controlled drug delivery besides the more conventional ones such as liposomes, lipid emulsions and polymeric particles.[1] They are very similar to emulsions but they are different in the lipid nature: the liquid lipids used in emulsions are replaced by a solid lipids at body temperature. Due to low mobility of drugs in the solid carrier, the formulations possess sustained release properties. Basically, the lipids used include high-melting point triglycerides, complex glycerides mixtures, fatty acids, steroids or waxes. Entrapment efficiency, loading capacity, and delivery of drugs have been reported for different application routes such as oral, parenteral, pulmonary, ocular, and topical.[2] Lipid micro- and nanoparticles are produced by several methods extensively described in the literature like high pressure homogenization,[3] microemulsion,[4] solvent emulsification-evaporation,[5] solvent emulsification–diffusion,[6] solvent injection,[7] W/O/W double emulsion,[8] high shear homogenization,[9] soap coacervation,[10] A promising technique for preparing micro- and nanoparticles is the electrospray, which is a slightly modified form of the electrospinning process that is used to produce polymer micro and nanofibers.[12] In electrospray, an electric field is applied to a polymeric solution contained in a syringe connected to a pump. The applied high voltage electric field forces the polymer solution to come out from the syringe in a jet form that eventually enables the formation of micro- and nanoparticles directly as a powder. The great advantage offered by electrospray over other commonly used methods is that it is a one-step process. Recently, electrospray was used to produce solid lipid micro- and nanoparticles.[13]

Over the last few years, several biotechnological peptides and proteins with therapeutic activity have been developed; these new biomolecules are characterized by high molecular weight, short biological half-life, poor absorption through membranes, and proteolitic degradation.[14] The possibility of incorporating these molecules in solid particles is interesting to obtain prolonged release pharmaceutical forms suitable to reduce most of these disadvantages. Due to their hydrophilic nature many peptides and proteins are poorly encapsulated into the hydrophobic matrix like lipids. A strategy to enhance the encapsulation efficiency is to increase drug hydrophobicity forming an ion pair with an hydrophobic counter ion.[15] Hydrophobic ion pairing (HIP) consists in the stoichiometric replacement of a polar counter ion with a lipophilic molecule of similar charge. HIP method is interesting in the field of protein and peptide delivery.[16] The enhanced hydrophobicity of the complex allows to dissolve greater amount of proteins and peptides in organic solvents usually used for the preparation of lipidic and hydrophobic polymeric particles.[17,18] The aim of this study was to produce insulin-loaded lipid microparticles using the electrospray procedure. Insulin was selected as a model peptide for its well known physiochemical

characteristics, moreover the insulin=SDS complex is one of the more extensively studied and characterized ion pair.[19]

MATERIALS

Palmitic acid (PA), stearic acid (SA), 1-propanol, Pluronic F127 (PL), and insulin from bovine pancreas (5733.49 Da) were purchased from Sigma (St. Louis, MO, USA). Ethylcellulose (EC, 64000 Da), sodium dodecyl sulfate (SDS) was from Fluka (Milan, Italy). Distilled water was purified using Milli-Q system (Millipore, Bedford, MA,USA); all chemicals were used without further purification or other processing.

METHODS

Preparation of Insulin/SDS Complex

The insulin=SDS complex was prepared by HIP method.[19] 10 milligrams of insulin were dissolved in 10mL of acidified water (pH 2.5). Then SDS was slowly added at 6:1 SDS=insulin molar ratio. The cloudy solution was centrifuged (Rotofix 32, Hettich Zentrifugen) at 10,000 rpm for 10 minutes at room temperature. The supernatant was removed and the white precipitate was washed with acidified water (pH 2.5) and water, lyophilized and stored at 5_C before further use.

Preparation of the Solutions for Electrospray

1-Propanol solutions containing PA in different concentrations (4.5-10-20%) or SA (4.5%) in presence of ethylcellulose (0.5-1%) or Pluronic F127 (0.5-1%) were prepared at 20°C or 40°C under magnetic stirring. Insulin or Insulin/SDS complex (0.1% of insulin) was added to the propanol solution to prepare drug loaded particles.

Experimental Electrospray Set-Up and Particle Preparation

The experimental set-up consisted of a 2.5mL syringe connected to an infusion pump (KDS 100, Biological Instruments, Varese, Italy). A Teflon capillary connected the syringe to a metal tip (ID 0.6 mm). An electric field was applied (GAMMA High Voltage Research, Ormond Beach, FL, USA) between the tip and the collecting screen (aluminium plate). The solutions were sprayed from the tip and, during jet's flight, the solvent gradually evaporated and the particles were deposited onto the collecting plate. The apparatus was hosted in a close transparent case at 20°C or 40°C. The following parameters for the electrospray process were used: 30 kV, distance metal tip-collecting plate 150 mm, and solution flow 5 ml/min.[20] All experiments were performed in triplicate.

Particles Size and Morphology

The mean diameter and polydispersities of the particles were determined in triplicate by photon correlation spectroscopy (90 Plus, Brookhaven Instrument, Holtsville, NY, USA). Measurements were performed at 25_C at an angle of 90_. All samples were obtained by dispersing the collected particles in filtered water using ultrasound. The particle morphology was examined by scanning electron microscopy (SEM; Leica Stereoscan 410, Wetzlar, Germany). A thin layer of particles was mounted on a copper stud, which was then sputter coated with gold (SCD 050, Leica, Wetzlar, Germany) for 60 seconds under vacuum at a current intensity of 60 mA. The gold coated particle layer was scanned using the accelerating voltage scanning of 20 kV.

Differential Scanning Calorimetry

Differential scanning calorimetry (DSC) measurements were performed by DSC7 (Perkin Elmer, Nortwalk, CT, USA). In each experiment, 2–3 mg of sample was sealed in the aluminium pan and scanned, with a similar empty pan as a reference, at a fixed heating rate (10°C/min) from 40°C to 80°C.

Encapsulation Efficiency

Encapsulation efficiency (EE) was calculated as the ratio between the amount of drug detected in washed lipid particles and the amount of drug used in the preparation. Insulin content within the lipid particles was determined in triplicate as follow: 5 mg of collected particles were washed three times with 1mL of pH 2.5 water to remove the adsorbed insulin. After centrifugation at 24000 rpm for 30 minutes (Allegra 64R centrifuge, Beckman Coulter, CA, USA), the solution was removed and the residue was dissolved in 0.5mL methanol, and then 0.5mL of 0.1M hydrochloric acid was added and mixed. After centrifugation (24,000 rpm, 30 minutes) the solution was injected for RP-HPLC measurements.

Determination of 1-Propanol Content

10-Propanol in PA-EC (10:1) particles was determined by gas-chromatography with a GC-Agilent 6890 equipped with quadrupole MS 5973 Network Agilent used in SIM mode; a weighted amount of collected particles was solubilized in methanol and injected into the GC system.

Circular Dichroism Analysis

Far-ultraviolet (UV) circular dicroism (CD) measurements of 0.2 mg/mL solutions of insulin standard solution and electrosprayed insulin were performed using a J820 spectral polarimeter

(Jasco, Gross-Umstadt, Germany) with the following settings: cell path length 0.2 mm, wavelength range of 260 to 190 nm, bandwidth 2 nm; speed 20nm/min, time constant 4 seconds. Each spectrum was obtained as an average of 5 repeated scans and corrected for solvent baseline at room temperature.

Mean residue ellipticity was calculated from the expression:

$$[\theta_\lambda] = \theta_\lambda M_0/C\ l;$$

where θ_λ is the observed ellipticity at wavelength λ, Mo is the mean residue molecular weight (which is for insulin 112),[21] C is the protein concentration (g/mL), and l is the optical path length (cm).

Insulin concentration was determined by RP-HPLC at the parameters subsequently reported.

In Vitro Drug Release

Insulin release was evaluated by suspending 10 mg of insulin-loaded microspheres in 10 ml of 0.05M aqueous phosphate buffer at pH 7.4. Triplicate samples for each batch were placed in a thermostatic bath at 37°C. At scheduled time intervals 500 ml aliquots of release medium were withdrawn and replaced with the same volume of fresh medium. Samples were filtered through 0.5 mm filters and analyzed by RP-HPLC.

High Performance Liquid Chromatography

Insulin was determined by high performance liquid chromatography (HPLC) system consisting of PU-1580 HPLC pump (Jasko, Gross-Umstadt, Germany), 2050 variable wavelength ultraviolet detector (Varian, Santa Clara, CA, USA) and a C-R6A integrator (Shimadzu, Duisburg, Germany). The quantitative determination of insulin was performed by a reverse-phase HPLC chromatography on Ultrasphere 5 mm C18 column (250mm×4.6 mm) (Beckman, Coulter, CA, USA). The mobile phase was a mixture of 1% sodium sulphate solution adjusted to pH 2.3 with phosphoric acid and acetonitrile (71/29 v/v). An injection volume of 20 ml and a flow rate of 1mL/min were used. The detection was performed at 220 nm.

RESULTS AND DISCUSSION

The preparation of lipid micro- and nanoparticles using the electrospray process and their improvement by the addition of a second component (stabilizer) has been reported in our previous work:[13] the formation of spherical particles lower than 1 mm with a narrow diameter distribution

was obtained by spraying a pentanolic solution of SA or PA (4.5%) and EC (0.5%) under an electric field of 30KV.

Starting from these considerations, pentanolic solution containing 4.5% SA, 0.5% EC, and 0.1% insulin were jetted in the same operative conditions: particles of different shapes, ranging from fragments to rounded particles, and a high percentage of degradation products were obtained.

Preliminary studies using different solvents were done to improve both particle morphology and insulin stability. Different shapes, similar to those obtained using 1-pentanol, but no peptide degradation products were observed using 1-propanol. A potential disadvantage of electrospray for aqueous solution is that, as the liquid evaporates in these highly charged droplets, the electric field around the particles intensifies. This field may interfere with protein conformation, yielding denaturation and loss of biological activity, well before even more dramatic effects, such as fragmentation, occur. In favor of the electrospray approach, on the other hand, is the fact that the droplets produced are sufficiently small that complete solvent evaporation over milliseconds occurs. Consequently, there is no risk of thermally damaging dissolved labile molecule.[22] As the conductivity of the propanolic and pentanolic solutions and the heat of vaporization of the solvents[23] are similar, the electric field is the same, the insulin stability was related to the different droplet temperatures related to the different boiling point of the solvents (1-propanol bp 97°C, 1-pentanol bp 136°C).

Low EEs (about 20%) were obtained, probably because of insulin separation and adsorption on lipid particle surface. As insulin is an hydrophilic molecule, it was difficult to dissolve completely the drug in the electrospray solution. Moreover, during rapid evaporation of the solvent, significant portion of the dissolved drug remained on the particle surface because of the low diffusion rate of such molecule in liquid solvent due to its large molecular weight. Hydrophilic peptide was then converted into lipophilic insulin/SDS.[19] Insulin complexed with SDS at an approximately stoichiometric ratio, and significantly increased both its solubility in 1-octanol and partitioning into 1-octanol in an octanol/water system. Moreover, it was shown that HIP complexes display enhanced ability to cross the membranes and increased stability. In addition, insulin in the complex retained its native secondary and tertiary structures, and also showed in vivo bioactivity comparable to free insulin in rats.[19] The effect of the complex on the morphology of 4.5% SA and 4.5% PA particles is reported in Figure 1: Particles varied from irregular shapes to spheres. Hollow particles were obtained using 4.5% PA and 0.5% PL: these particles should have a great potential for pulmonary drug delivery applications due to their large surface area and low mass density. Electrospray is a complex process and an accurate estimation of particle size and morphology beforehand is very difficult. Many parameters can influence the transformation of solution into

particles as previously reported for polymers.[24] These parameters, considering the process parameters constant, include the properties of the solution, such as viscosity, conductivity and surface tension, and the properties of the solute, such as solubility and molecular weigh. The addition of surfactant (such as PL) has the effect of reducing the surface tension of the organic solution enhancing the bending instability.[25] Based on SEM images, there was an improvement in morphology in the presence of 0.5% PL, while, no significant improvement with even higher concentration of PL (data not reported) was observed. This was most probably due to the fact that maximum surface concentration has been reached.

EC is a large macromolecule, the diffusion rate of such molecule in liquid solvent would be very low. As the droplet shrank due to the loss of solvent through surface evaporation, EC concentration near the surface increased and a thin shell of solid EC would be formed on the droplets surface. When the concentration of the lipid was low (4.5%), the particles would be structurally weak, so the shell would fragment into individual pieces. The next set of experiments was conducted to study the effect of lipid concentration on particle morphology. PA was selected considering SEM images shown in Figure 1 and the higher solubility in propanol (about 12% at 20°C, 25% at 40°C) compared to SA (5% at 20°C, 12% at 40°C).

Figure 2 shows the effect of PA and stabilizer concentrations on the particles morphology. Particles of uniform rounded shape within a narrow range of size distribution were obtained, except for 20% PA-1% EC which produced agglomerates. In the present case, fibers and interconnections were not formed: lipid structure and stabilizer concentrations were not suitable to give sufficient chain entanglements, as reported for polymers.[26] The probable reason may be that the Rayleigh forces overcome the viscous forces leading to the formation of particles. The mean particle sizes and polydispersity, determined by photon correlation spectroscopy, are reported in Table 1. It was observed that increasing PA concentration in the absence and in the presence of insulin resulted in increasing particles size. In this case, a high concentration of lipid could be established on the surface of the droplet with less solvent evaporation. Therefore, a surface shell with larger diameter was formed and resulted in a larger final particle size. Unexpected high polydispersity, compared to SEM results, was measured probably due to the presence of some aggregates because of the difficulty to disperse the electrosprayed particles in the suspending medium.

The DSC traces of PA raw material and insulin-loaded PA-EC particles are reported in Figure 3; loaded PA-EC particles and PA bulk material thermograms are similar and a single endothermic event of the insulin-loaded particles at 62°C was observed.

The amount of residual 1-propanol in loaded PA-EC particles determined by gas-chromatography was about 0.1 mg per gram of particles, confirming the data previously obtained with SA

particles.[13] The suggested limit of residual 1-propanol listed by the FDA guidance for patient safety is 0.5% w=w,[27] consequently the lipid particles can be considered to have low toxic potential. In Figure 4, EEs of insulin are shown. EEs, above 65% for all formulations and in particular more than 90% for 20% PA, were obtained. As reported for other lipophilic drugs soluble in solvents used for the production of polymeric particles,[28] phase separation was difficult to take place, and the drug was apt to remain inside the polymer where there was enough solvent left. Thus, when the polymer dried, the drug was encapsulated inside the particles.

Structural integrity of insulin after encapsulation into fatty acid particles was evaluated by circular dichroism spectroscopy. The Far-UV CD spectra of insulin in 0.01M HCl and insulin extracted from the lipid particles (Figure 5) show two minima at 208 and 222nm, which is typical of predominant a-helix structure proteins:[21-29] no significant difference in both CD spectra and mean residue ellipticity values were observed. These data indicate that electrospray did not induce significant changes in α-helix/β-sheet insulin content and insulin likely preserved its intrinsic conformation after encapsulation into fatty acid microparticles.

The in vitro release profiles of insulin and insulin/SDS from PA particles are reported in Figure 6. It is interesting to note that the complex loaded particles showed an in vitro sustained release for 24 hours after an initial burst (about 10%). This behavior was different from the one obtained using native insulin. In this case, during electrospraying, if there are limited physical interactions between the drug and the matrix, then the majority of the drug will likely be localized on the surface of the particles. In such an arrangement, drug molecules on the particle surface can be easily washed away in aqueous release medium, thus resulting in a large initial burst at short time and minimum sustained release at longer time. When the hydrophobic complex was used, it is conceivable that some drug molecules can be trapped in the hydrophobic lipid particles during electrospray. Such a structure is consistent with the sustained drug release behavior seen.

Even if PL is more water-soluble than EC, no appreciable differences were observed in the drug release profiles between PA containing EC or PL and this was attributed to the low percentage of the stabilizers in lipid particles, not able to influence the release behavior.

CONCLUSIONS

Lipid-based microspheres of about 1 mm were prepared in a single step directly in powder form by electrospray. Insulin was entrapped into the particles with high encapsulation efficiency by the formation of an ion-pair. Far-UV CD spectra indicated that electrospray did not induce a significant change in the secondary insulin structure. This study demonstrates that electrospray represents a

viable new alternative for preparing peptide-protein loaded lipid based microparticles for drug prolonged release.

This technique appears efficient, versatile and easy to be implemented.

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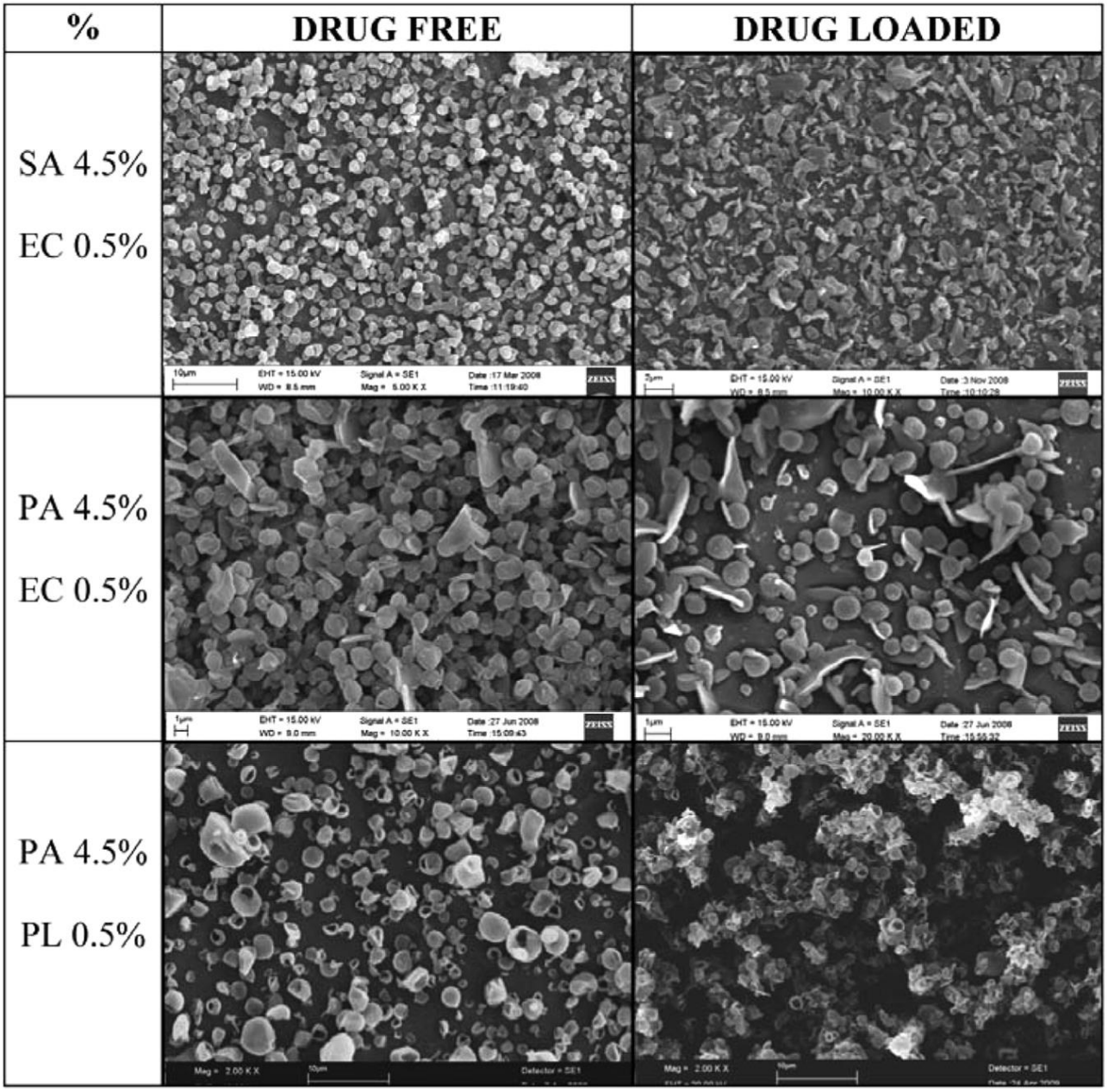

FIG. 1. Scanning electron micrographs of SA-EC (4.5:0.5), PA-EC (4.5:0.5), and PA-PL (4.5:0.5) SLN.

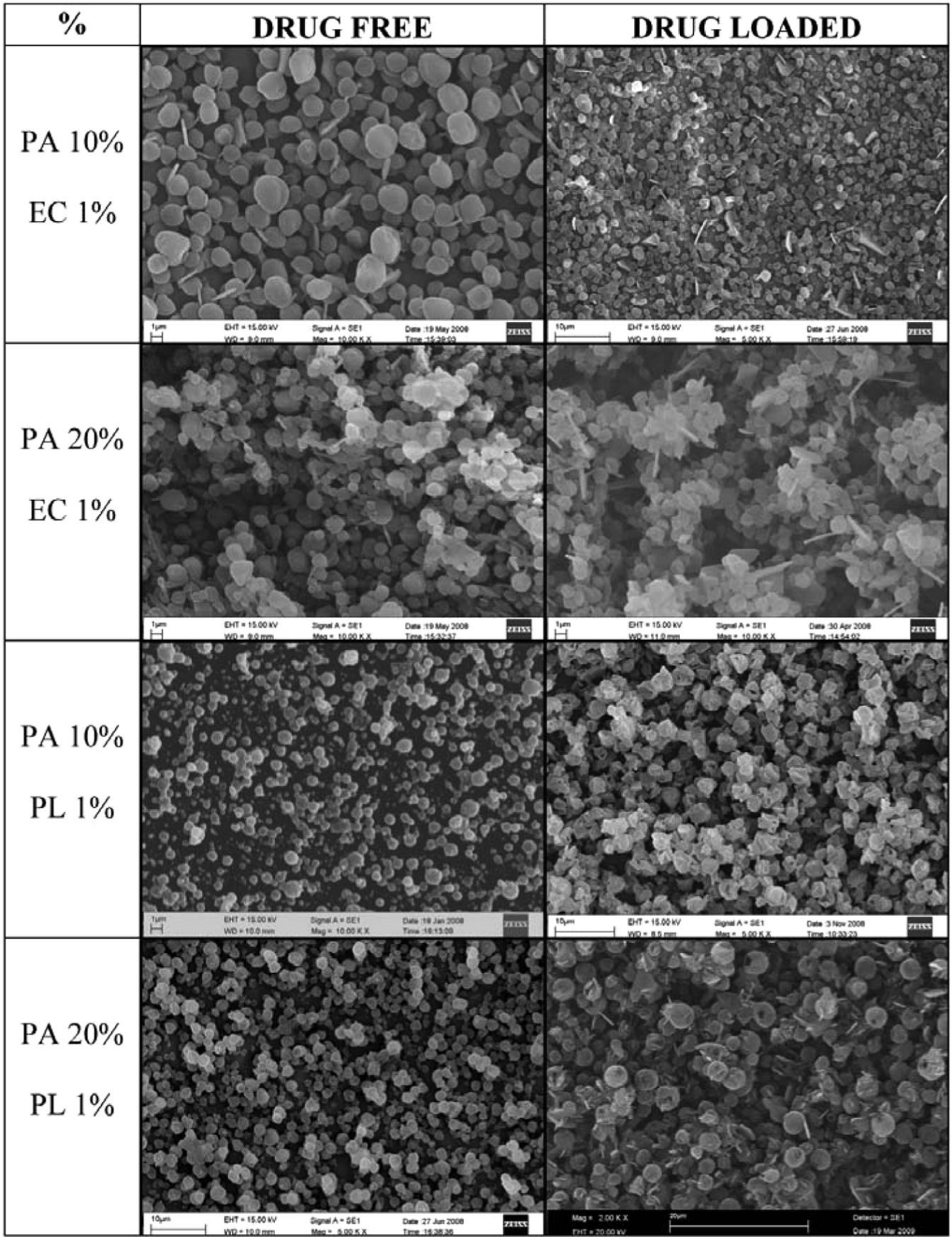

FIG. 2. Scanning electron micrographs of PA-EC (10:1 and 20:1) and PA-PL (10:1 and 20:1) SLN.

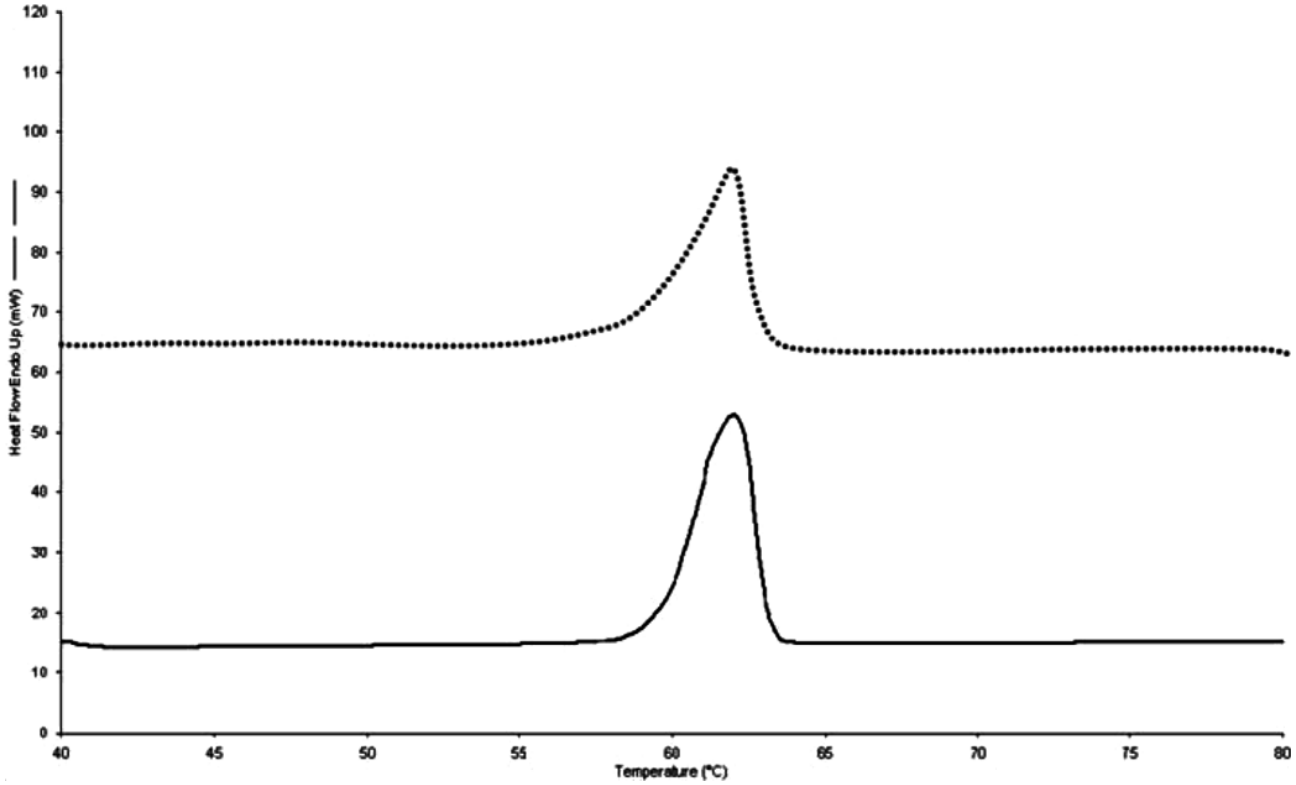

FIG. 3. DSC thermograms: (full line) PA, (dashed line) insulin-loaded PA particles.

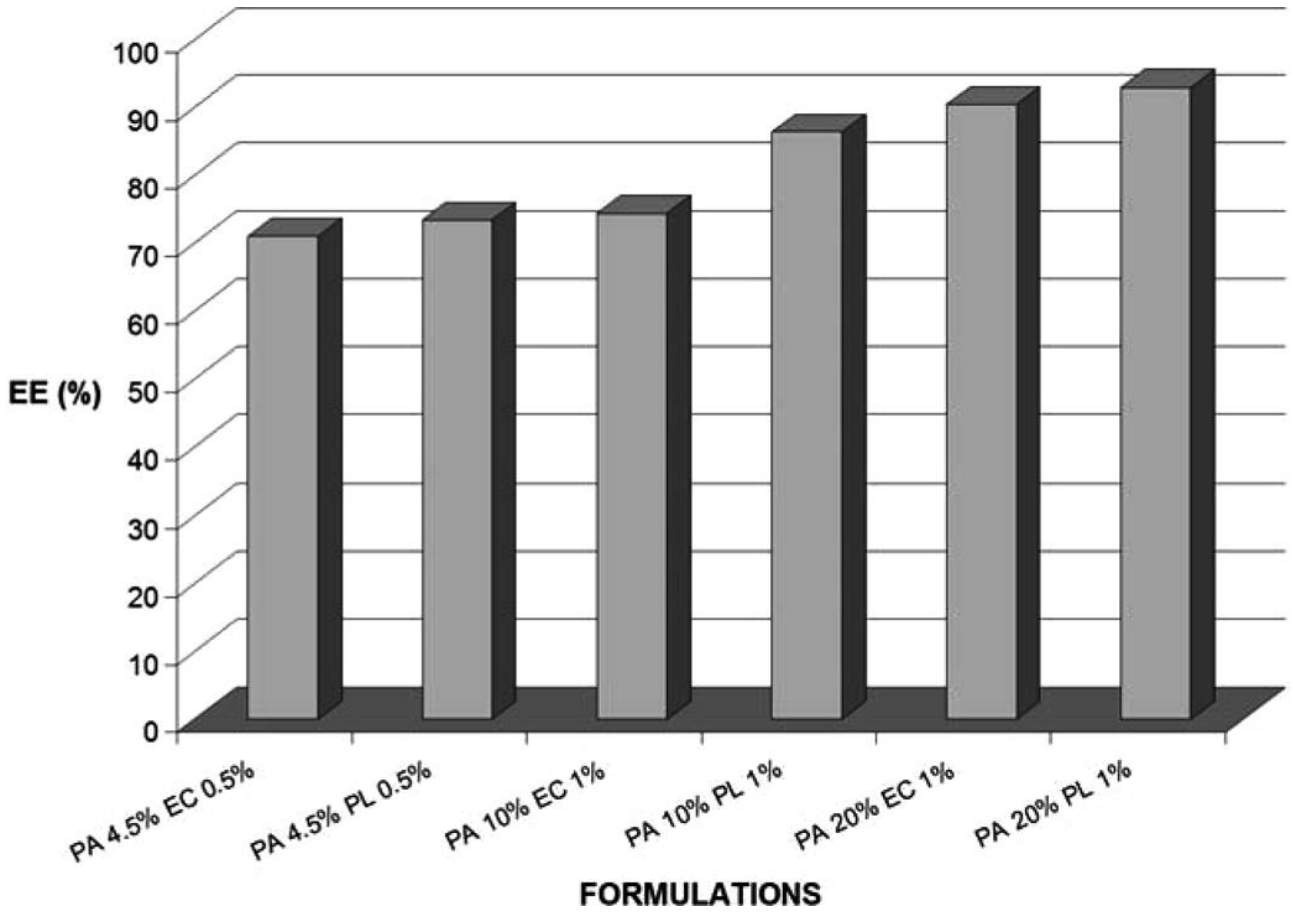

FIG. 4. Insulin encapsulation efficiency (EE) in SLN.

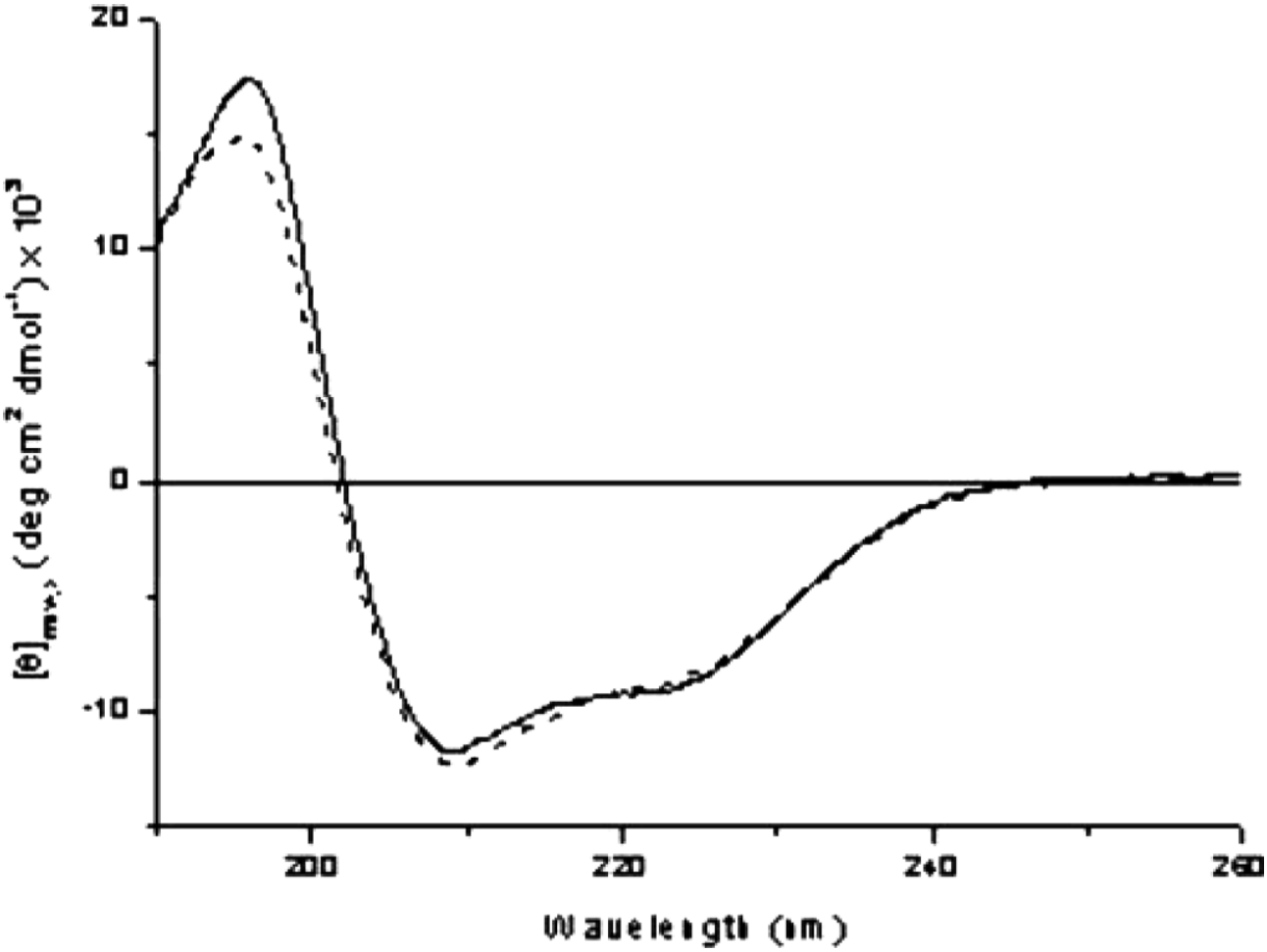

FIG. 5. Far-UV CD spectra of insulin standard in 0.01 HCl solution (full line) and electrosprayed insulin (dashed line).

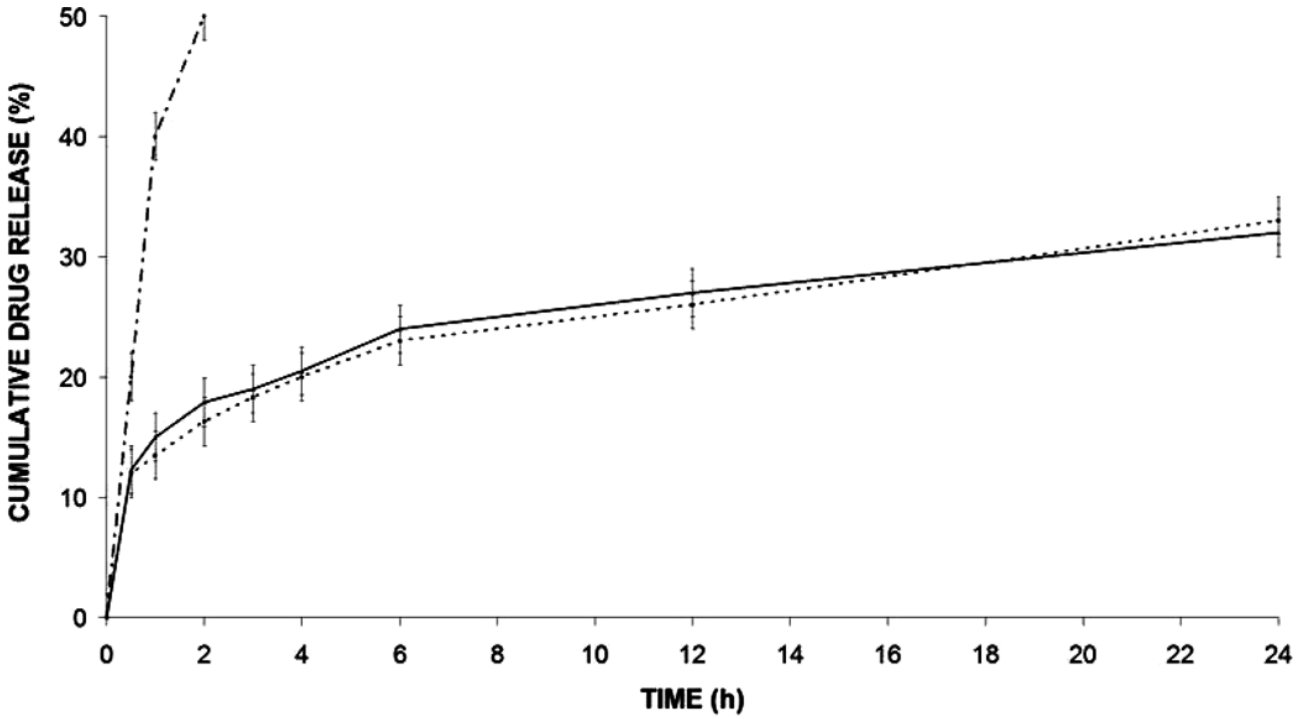

FIG. 6. In vitro release profile of free insulin (dashed-pointed line) and insulin from PA-EC (10:1) particles (dashed line) and PA-PL (10:1) particles (full line) in pH 7.4 phosphate buffer.

TABLE 1
Mean particle size and polydispersity index of PA-particles

Formulation	Drug free	Drug loaded
PA 10% EC 1%	835.0±91.2 (0.228)	900.4±31.9 (0.214)
PA 20% EC 1%	1043.8±69.2 (0.234)	1171.1±37.6 (0.223)
PA 10% PL 1%	958.2±31.3 (0.241)	965.0±35.2 (0.248)
PA 20% PL 1%	1128.1±28.6 (0.213)	1198.8±32.4 (0.207)