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## **Preparation of solid lipid nanoparticles from W/O/W emulsions: preliminary studies on insulin encapsulation**

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A method to produce solid lipid nanoparticles (SLN) from W/O/W multiple emulsions was developed applying the solvent-in-water emulsion-diffusion technique. Insulin was chosen as hydrophilic peptide drug to be dissolved in the acidic inner aqueous phase of multiple emulsions and to be consequently carried in SLN. Several partially water-miscible solvents with low toxicity were screened in order to optimise emulsions and SLN composition, after assessing that insulin did not undergo any chemical modification in the presence of the different solvents and under the production process conditions.

SLN of spherical shape and with mean diameters in the 600-1200 nm range were obtained by simple water dilution of the W/O/W emulsion. Best results, in term of SLN mean diameter and encapsulation efficiencies were obtained using glyceryl monostearate as lipid matrix, butyl lactate as a solvent and soy lecithin and Pluronic®F68 as surfactants. Encapsulation efficiencies up to 40% of the loaded amount were obtained, owing to the actual multiplicity of the system; the use of multiple emulsion-derived SLN can be considered an useful strategy to encapsulate a hydrophilic drug in a lipid matrix.

**Keywords:** Solid lipid nanoparticles (SLN), insulin, W/O/W emulsions.

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## 1. Introduction

The development of colloidal carrier systems in drug administration has attracted increasing attention during recent years as innovative strategy to overcome frequent therapy failures due to unpredictable bioavailability of drugs when administered by the conventional routes and in the conventional dosage forms. The most investigated systems are simple and multiple emulsions, liposomes, micelles, micro and nanoparticles based on synthetic polymers or natural macromolecules (Müller 1998a).

Particularly, peptides and protein drugs represent a very important class of therapeutic agents, owing to the understanding of their role in physiology and pathology as well as to the advances in biotechnology and genetic engineering. Unfortunately, they are characterised by a short biological half-life, being easily degraded by proteolytic enzymes and, moreover, most peptides poorly pass through biological barriers due to their poor diffusivity and low partition coefficient.

In recent years, the attention of many researchers has focused on solid lipid nanoparticles (SLN) as alternative drug carriers to polymer nanoparticles (Müller 1998b; Mehnert 2001; Müller et al. 1995), as it has been claimed that they can combine many advantages and avoid most disadvantages of other colloidal carriers. The solid lipids used as a matrix material for drug delivery are generally well tolerated by the body, (e.g. glycerides composed of fatty acids which are present in the emulsions for parenteral nutrition).

The possibility of incorporating peptide drugs in lipid particles could exert a protection of the drug against degradation by the proteolytic enzymes present in the gastrointestinal apparatus. However, the use of lipids as matrix materials for sustained-release formulations for peptides and proteins has been reported only by a few Authors (Reithmeier et al. 2001; García-Fuentes et al. 2002), owing to the hydrophobic nature of lipid matrix that can be more appropriate to incorporate lipophilic drugs rather than hydrophilic proteins.

In a previous work (Trotta et al. 2005), solid lipid insulin-loaded microparticles were produced by the solvent-in-water emulsion-diffusion technique, described in literature only for

polymeric micro-and nano particles, using isobutyric acid, a partially water-miscible solvent with low toxicity and high insulin-solubilization capacity. Later (Battaglia et al. 2007), the production process and the insulin encapsulation efficiency were improved using isovaleric acid: *in vivo* experiments proved that insulin bioactivity was maintained in SLN. The solvent-in-water emulsion-diffusion technique involves the emulsifying of an organic solution of lipid and drug into a continuous aqueous phase in which the solvent is partially soluble and the further water-dilution of the resulting O/W emulsion (Trotta et al. 2003). Upon transferring the transient O/W emulsion into water, the lipophilic material dissolved in the organic solvent solidifies instantly due to the diffusion of the organic solvent from the droplets to the continuous phase. Using optimised formulations, insulin-loading SLN were obtained.

Considering the hydrophilic nature of insulin, some Authors (Cunha et al. 1997a) vehicled it in the inner aqueous phase of stable double W/O/W emulsions which were able to protect insulin against enzymatic degradation. They also described the oral administration in rats of two insulin-containing multiple W/O/W emulsions composed of soybean oil or medium-chain triglycerides (Cunha et al.1997b): single administration in normal and diabetic rats and short-term treatment in diabetic rats showed that in these animals small amount of biologically active insulin were absorbed from these emulsions. Some Authors (Couvreur et al.1997) developed a preparation process for its encapsulation consisting in the formulation of a W/O/W multiple emulsion containing insulin in the inner aqueous phase, poly(lactic-co-glycolic acid) in a volatile organic solvent, and the emulsifier in the outer aqueous phase: the evaporation of the solvent from the W/O/W emulsion allows the formation of solid drug-loaded microspheres. The literature reports also the formulation of PEG 2000-coated tripalmitin nanoparticles loading insulin, precipitated from multiple emulsions by evaporation of the solvent (García-Fuentes et al. 2002).

The aim of the present study was to develop and characterise SLN, intended for loading insulin, using glyceryl monostearate (GMS) and cetyl palmitate (CP) as matrix materials, and low-

toxicity solvents as oil phases, obtained by solvent removing methods from W/O/W multiple emulsions.

## 2. Materials and methods

### 2.1. Materials

Bovine insulin ( $M_w=5807$ ), taurodeoxycholic acid sodium salt (TDC), trifluoroacetic acid (TFA) propyl acetate (PA), *iso*-propyl acetate (IPA) and lysozyme were from Sigma Chemical Co. (Milano, Italy). Cetyl palmitate (CP) and glyceryl mono-stearate (GMS) were from Goldschmidt (Essen, Germany). Butyl lactate (BL) ethyl acetate (EA), methylene chloride (MC), Tween®20 and Tween®80 were from Fluka (Buchs, Switzerland). Pluronic®F68 was from Serva (Heidelberg, Germany). Simulsol®165 [PEG-100 stearate and glyceryl stearate] was from Seppic (Paris, France). Soya lecithin (Epikuron®200) was from Lucas Meyer (Hamburg, Germany). A21-desamido insulin was prepared by storing bovine insulin in 0.01 M HCl at 50°C for 48 h. Distilled water was purified using a Milli-Q system (Millipore, Bedford, MO). All other chemicals were analytical grade and used without further purification.

### 2.2. GMS and CP apparent solubility

Increasing amounts of GMS or CP were added to 1 ml of the chosen solvent or of water-saturated solvent: the resulting samples were sealed and stirred at 25±1°C or 50±1°C for 12 h. MC, BL, EA, IPA, PA were tested as solvents. The lipid apparent solubility was determined considering the highest lipid amount which gave a transparent solution on visual observation.

### 2.3. Insulin apparent solubility

The solubility of insulin at 25±1°C and 50±1°C in BL, EA, IPA, PA and in water-saturated BL, EA, IP, PA was evaluated by determining the minimum amount of solvent required to dissolve a known amount of drug at the chosen temperature.

### 2.4. Insulin heat stability

Preliminary experiments on insulin stability in the presence of solvents and surfactants tested in SLN formulation were performed in order to assess the optimal experimental conditions. A 0.6% w/v insulin solution in 0.01 M HCl was prepared in the presence of: EA, IPA, PA and BL, at 3.0% w/v, and of Tween®20, Tween®80, Epikuron®200, Simulsol®165, TDC. Samples were placed in

a thermostatic water bath (Tecno Galenica, Milano, It) and kept at  $50 \pm 1$  °C. At scheduled times, an aliquot was withdrawn, filtered and analysed by RP-HPLC and SE-HPLC.

### 2.5. *Insulin analysis*

RP-HPLC and SE-HPLC were used for insulin determination. The chromatograph was equipped with a Shimadzu HPLC system (Shimadzu, Milano, Italy), set at 220 nm.

For the RP-HPLC the analytical conditions were as follows: column: C18 25x0.4 cm (Beckman Ultrasphere); mobile phase: 0.1 M  $\text{Na}_2\text{SO}_4/\text{CH}_3\text{CN}$  (72/28) brought to pH 2.3 with  $\text{H}_3\text{PO}_4$ ; flow: 1  $\text{ml min}^{-1}$ ; retention times: 11.0 and 12.5 min for insulin and A21-desamido insulin, respectively. A calibration graph was constructed in the  $0.8 \mu\text{mol l}^{-1} - 20 \mu\text{mol l}^{-1}$  range (8 points, each determined in quadruplicate). The linearity of the calibration graph was demonstrated by the value (0.9981) of  $R^2$  coefficient of the regression equation:  $y = 6.97 \cdot 10^{10}x - 16176$ . The LOQ, defined as the lowest insulin concentration in the curve that can be measured routinely with acceptable precision and accuracy was  $1.0 \mu\text{mol ml}^{-1}$ ; the LOD, defined as the lower detection limit was  $0.40 \mu\text{mol l}^{-1}$  (signal to noise  $> 2.0$ ).

For the SE-HPLC, the analytical conditions were as follows: column: TSK gel G2000SWXL (Tosoh Bioscience, Stuttgart, Germany); mobile phase:  $\text{CH}_3\text{CN}/0.05\% \text{ TFA}$  10:90; flow: 1  $\text{ml min}^{-1}$ ; retention time: 7.7 min. Lysozyme ( $M_w=14.3$  kDa retention time 6.8 min) was used as external standard to exclude dimerisation products. The linearity of the calibration graph was demonstrated by the value (0,9998) of  $R^2$  coefficient of the regression equation:  $y = 9.86 \cdot 10^{10}x - 415\ 089$ . The LOQ was  $0.89 \mu\text{mol l}^{-1}$ ; the LOD was  $0.25 \mu\text{mol l}^{-1}$  (signal to noise  $> 2.0$ ).

### 2.6. *Insulin aggregation induced by water-organic solvent interface*

Insulin aggregation and fibrils formation were monitored by turbidity measurements as described in literature (Kwon et al. 2001).

8.5 mg of Zn-crystalline insulin powder were dissolved in 1.0 ml 0.01 M HCl: then 2.5 ml of a twice-concentrated phosphate-buffered saline solution (0.02 M phosphate, 0.29 M NaCl, 1.0 mM EDTA) was added dropwise. pH was then adjusted to 7.4 adding 0.1 M NaOH. The sample was



then diluted to 5.0 ml with bidistilled water, filtered through a 0.22  $\mu\text{m}$  filter (Millipore). 500  $\mu\text{l}$  of the resulting solution were placed in a quartz cell (1.0 cm path length) in the presence of 1.5 ml of the solvent under study. Insulin aqueous solution was placed first in the bottom of the cuvette and the solvent was then added on the top, unless for MC, according to the different reciprocal densities. The percentage of transmittance at 350 nm at 25°C was recorded as a function of time for 120 min. Decreased transmittance was associated with increased aggregation at the solvent-aqueous solution interface. Solvent under study were: EA, IPA, PA, BL, MC.

### 2.6. *Insulin apparent partition coefficient*

The apparent partition coefficient ( $P_{\text{app}}$ ) of insulin was determined by dissolving a weighted amount of insulin in 0.1 N HCl-saturated solvent (EA, PA, IPA, BL, MC). Each solution was added to 2 ml solvent-saturated water. The mixture was shaken for 5 minutes and then stored for 12 h at 50 $\pm$ 1°C. Insulin concentration in the acid aqueous phase was determined by size exclusion chromatography (SE-HPLC) and the apparent partition coefficient was calculated.

### 2.7. *SLN preparation*

2.7.1. *W/O/W multiple emulsions development and SLN precipitation.* W/O/W multiple emulsions were prepared by a two-step procedure (Florence 1982), involving a step in which the primary W/O emulsion is obtained followed by a step in which the primary emulsion is slowly added to a continuous aqueous phase. The solvent under study (MC, PA, IPA, EA, BL) and water were mutually saturated to ensure initial thermodynamic equilibrium of both liquids.

Emulsions 1-3 - Insulin was dissolved in solvent-saturated 0.01 M HCl at a 8.0 mg ml<sup>-1</sup> concentration: 500  $\mu\text{l}$  of this solution, as inner aqueous phase, was emulsified at 50 $\pm$ 1°C at 2400 rpm (Ultra Turrax T-25 Ika Labortechnik) with 2.0 ml of water-saturated organic solvent, in which the lipids (GMS:CP 2:1 w/w) were previously dissolved. The primary W/O emulsion obtained was then gently poured (100  $\mu\text{l min}^{-1}$ ), under vigorous mixing, at 50 $\pm$ 1°C into 0.9 ml of the solvent-saturated outer aqueous phase containing the hydrophilic surfactant (2.0% w/w Pluronic® F68 solution in 10 mM phosphate buffer at pH 5.21) to give a W/O/W emulsion, which was kept at

50±1°C for 1 hour.

Emulsions 4-6 - Insulin was dissolved in solvent-saturated 0.01 M HCl at a 23.0 mg ml<sup>-1</sup> concentration: 100 µl of this solution, as inner aqueous phase, was emulsified at 50±1°C at 2400 rpm (Ultra Turrax T-25 Ika Labortechnik) with 1.0 ml of water-saturated organic solvent, in which the lipid (GMS) and lecithin were previously dissolved. The primary W/O emulsion obtained was then gently poured (100 µl min<sup>-1</sup>), under vigorous mixing, at 50±1°C into 3.0 ml of the same outer aqueous phase described for emulsions 1-3, to give a W/O/W emulsion, which was kept at 50±1°C for 1 hour.

Emulsion 7 - Insulin was dissolved in solvent-saturated 0.01 M HCl at a 23.0 mg ml<sup>-1</sup> concentration: 200 µl of this solution, as inner aqueous phase, was emulsified at 50±1°C at 2400 rpm (Ultra Turrax T-25 Ika Labortechnik) with 1.0 ml of water-saturated organic solvent, in which the lipid (GMS) and lecithin were previously dissolved. The primary W/O emulsion obtained was then gently poured (100 µl min<sup>-1</sup>), under vigorous mixing, at 50±1°C into 1.0 ml of the same outer aqueous phase described for emulsions 1-3, to give a W/O/W emulsion, which was kept at 50±1°C for 1 hour.

SLN were precipitated at 25±1°C by quickly adding water (15.0 ml) to 4.0 ml of each W/O/W emulsion to extract the solvent in the continuous aqueous phase according to its fair water solubility. After stirring at 600 rpm for 1 hour, the suspension was centrifuged at 24 000 rpm for 30 min (Allegra™ 64R Beckman Coulter); the supernatant was then removed and SLN were washed twice with bidistilled water and three times with 0.01 M HCl to remove insulin adsorbed into SLN surface. SLN were then dried under vacuum. Each dispersion was prepared thrice and submitted to the following characterizations.

## *2.8. Emulsion and nanoparticles characterisation*

*2.8.1. Optical microscopy.* W/O primary emulsions and W/O/W multiple emulsions were observed in optical microscopy at 50±1°C (Leitz Labovert) without any further dilution. Mean diameters were evaluated on the basis of the observation of at least 200 droplets (Motic Images Plus 2000).

2.8.2. *Laser Light Scattering*. The particle size of the SLN dispersions were determined by the laser light scattering technique LLS (Brookhaven, New York, USA). Measurements were obtained at an angle of 90°. Scattering intensity data were analysed by a digital correlator and fitted by the method of inverse Laplace transformation. The dispersions, opportunely diluted with water for size determination, were examined just after preparation and after re-dispersion of the SLN centrifuged at 24000 rpm for 30 min. Analyses were repeated on three samples of SLN precipitated separately from W/O/W emulsions: each measurement was repeated thrice.

2.8.3. *Differential scanning calorimeter (DSC) measurements*. DSC was performed with a Perkin-Elmer differential calorimeter (Norwalk, CT, USA). Lipid bulk material, SLN suspensions and an Epikuron 200 liposomes suspension were placed in conventional aluminium pans. Experimental conditions were as follows: scan speed 5°C min<sup>-1</sup>; temperature range 10-80°C.

### 2.9. *Insulin encapsulation efficiency*

A weighed amount of dry SLN, obtained as described in section 2.7.2, was dissolved in methanol and 0.01 M HCl was added to dissolve insulin. After evaporating methanol under a N<sub>2</sub> stream, the suspension was centrifuged at 15 000 rpm for 10 min (Eppendorf, Centrifuge 5417) and the solution was analysed for insulin content by RP-HPLC and SE-HPLC.

The results are expressed as encapsulation efficiency, i.e. the ratio between the actual and theoretical insulin loading percent, and as milligrams of insulin loaded *per* gram of SLN

## 3. Results and discussion

The solvent-in-water emulsion diffusion technique was proposed to obtain SLN loading insulin, which was chosen as hydrophilic, water unstable protein with very low oral bioavailability. In previous works (Trotta et al. 2005, Battaglia et al. 2007) several insulin-loaded SLN with high encapsulation efficiency were obtained by water dilution of O/W emulsions containing isobutyric or isovaleric acid as solvents and GMS or CP as lipids. The assessed stability of insulin during the

formulation of SLN, as well as the significant protection towards its proteolytic degradation, also confirmed by the maintenance of its biological activity after administration *in vivo*, were a stimulant preamble to further develop the research aimed to propose insulin-loaded SLN for oral administration. Considering that the most important pre-requisite to obtain a sufficient SLN loading capacity of insulin is its adequately high solubility in the lipid melt, and that the solubility of insulin in most commonly employed solvents and lipids is quite low, an alternative way to obtain insulin-loaded SLN is the development of W/O/W emulsions containing the peptide in the internal aqueous phase (Garcia-Fuentes et al. 2002).

Several SLN dispersions were obtained by water dilution of W/O/W emulsions prepared with some of the solvent tested. Initial experiments were intended to define the optimal conditions for the production of SLN by the double emulsion method, investigating several parameters such as volumes of aqueous and oil phases, surfactant type and concentrations.

The solvents under study were chosen for their low toxicity profiles and their fair water solubility (Table I). Moreover, in the experimental conditions BL, EA, PA, IPA showed fair solving capacity towards GMS and CP (Table II), which was particularly pronounced at 50°C; MC was used as reference solvent, as it has been widely described in the literature to prepare SLN from multiple emulsions (Garcia-Fuentes et al. 2002). These properties, together with the absolute lack (at 25°C) or very slight (at 50°C) insulin solving ability of BL, EA, IPA and PA make them particularly suitable to be employed as oil phase components of W/O/W emulsions (Table III). Moreover, to promote the dissolution of insulin in the inner aqueous phase of double emulsions, 0.01 M HCl was employed to formulate the primary W/O emulsion, being insulin fairly soluble in acidic media. Apparent solvent/0.01 M HCl partition coefficients of insulin were determined to evaluate the optimal experimental condition to obtain as high as possible peptide concentrations in the inner aqueous phase. The low values of apparent solvent/HCl partition coefficients of insulin were quite promising to avoid drug diffusion from the inner aqueous to the solvent phase of the multiple emulsions under study (Table IV).

A further prerequisite to successfully formulate insulin-loaded SLN is the maintenance of the chemical structure of the protein during the production process. It is well known that insulin in solution can undergo chemical and physical degradation (Brange et al. 1992), deamidation being the main degradation products. The influence of several surfactants and solvents on the stability of insulin was next verified: note that the term “stability” is here defined as the ability of insulin not to undergo transformation into A-21 desamido insulin, assessed by RP-HPLC, and into covalent aqueous soluble dimers, assessed by SE-HPLC. HPLC analysis of 0.01N HCl solutions kept at 50°C in the presence of different surfactants and solvents, as described in Methods section, were performed over time, and the results are reported in Figure 1. As can be noted, no or almost negligible chemical degradation of the peptide seemed to occur, suggesting that, for the short time required in production process, the above described procedures of SLN preparation and of insulin extraction from SLN did not negatively influence the chemical stability of the peptide. In an already mentioned experimental paper (Battaglia et al. 2007) the SEC chromatograms of an insulin standard solution and of SLN-extracted insulin were reported, where the lack of modification in peak shape, as well as the invariability of retention time confirmed the stability of the peptide during SLN preparation.

Figure 2 shows the aggregation rate of insulin for up to 2 hours at different solvent/water interfaces. Rapid aggregation occurred only in the presence of MC, as determined by the sharp decrease in % T at 350 nm vs. time, which decreased to 5% within 30 minutes. When BL, EA, IPA, PA were employed as solvents, no detectable change was noted in %T up to 2 hours. As a consequence of these results, BL, EA, IPA, PA were tested as solvents to formulate multiple emulsions, while MC was employed only as reference solvent widely used in the literature (García-Fuentes et al. 2002).

Several SLN dispersions, with mean diameters ranging from 500 to 2000 nm were obtained, deriving by dilution of W/O/W emulsions, under a standard stirring rate, of 4.0 ml of emulsion with an additional 15.0 ml of distilled water, just to extract all the solvent from the oil phase of the

double emulsion. In a first series of W/O/W emulsions, GMS was dissolved in MC, used as reference solvent cited in the literature, or in PA, IPA or EA chosen for their low toxicity; the amount of the inner aqueous phase was 4.5% w/w. SLN obtained by dilution of such emulsions revealed only a negligible encapsulation efficiency of insulin (data not reported). A second series of SLN (Table V, emulsions 1-3) was formulated using GMS and CP as lipids, BL, EA or PA as solvents, Tween®80 as emulsifier. To promote the dissolution of insulin in the inner aqueous phase, 0.01 M HCl was employed as aqueous phase of the primary W/O emulsion, and a 2.0% w/w Pluronic® F68 solution in 10mM pH 5.2 phosphate buffer as outer aqueous phase of the W/O/W emulsion, which contained 1.2 mg/ml insulin: the amount of the inner insulin-loaded aqueous phase was 14.9% w/w. The effective multiplicity of the resulting emulsions was confirmed by microscopic observation, as shown in Fig. 3: several small water droplets can be seen entrapped within an oil drop of emulsion n.1: mean diameters of the droplets ranged from 1 to 3 µm. Also the resulting SLN, analysed by LLS, presented mean diameters in the 1-3 µm range; the encapsulation efficiency was rather low, ranging from 5 to 10 % (4-8 mg insulin/g SLN) (Table VI) and most insulin was recovered in the outer aqueous phase, probably as a consequence of the diffusion of the peptide from the inner aqueous phase through W/O and O/W interphases.

Soya lecithin (Epikuron®200) was therefore successively, employed as a surfactant to substitute Tween®80, as it is known to contribute to give a certain rigidity to the interphase structure. Several W/O/W emulsions were prepared varying the percentages of the inner aqueous phase, of the lipid and of soya lecithin; the compositions of the emulsions which gave SLN producing the best results in term of mean diameters and encapsulation efficiency are reported in Table V (emulsions 4-6). The microphotographs of W/O/W emulsions 4 and 6, formulated with BL and PA as solvents respectively are reported in fig. 3: all emulsions showed a marked multiplicity, as many droplets were located within oil droplets. SLN obtained by simply dilution of emulsions 4, 5, 6 had mean diameters in the 700-1000 nm range, as confirmed by laser light scattering measurements (Table VI). As confirmed by LLS measurements and from encapsulation efficiency,

emulsion with BL as solvent had mean diameters around 600 nm and presented the highest encapsulation efficiency, which was higher than 40%. (Table VI), despite a moderately higher solvent action towards insulin than the other solvents under study. For this reason, the further formulation development was aimed to optimise only the formulation of SLN obtained by dilution of W/O/W emulsions containing BL as a solvent.

The possible formation of lecithin liposomes carrying insulin could be excluded according to the results of DSC analysis, which allowed investigating the status of the lipid particles: fig. 4 shows the DSC thermograms of GMS bulk material and Epikuron, liposomes and of GMS-Epikuron particle suspension. The peak location of GMS particles, slightly shifted towards lower temperature compared to that of the bulk material, is probably due to the presence of lecithin: on the other hand, no transition peak of lecithin liposomes is present, indicating that Epikuron is mostly located within lipid nanoparticles.

To improve the multiplicity of the double emulsion as well as to increase the interface's rigidity, Simulsol®165 was used to partly substitute Epikuron® as supplementary surfactant in the double emulsion. Consisting of a mixture of PEG-100 stearate and glyceryl stearate, it can be considered as a non ionic, self-emulsifying wax, especially designed for use in O/W emulsions. Several emulsions were obtained varying the percentage of oil and outer aqueous phases from 21 to 42% and from 40 to 70% respectively. In table V only the compositions of the best emulsion (n. 7) is reported, corresponding to the highest percentages of oil in a series of multiple emulsions. All W/O/W emulsions of this series showed actual multiplicity when microscopically observed and mean diameters below 1.0 µm; particularly, resulting SLN had mean diameters around 600 nm.

The highest encapsulation efficiency obtained in this series of SLN was almost 25% (in SLN n.7), corresponding to a loading of almost 12 mg insulin/g SLN, quite analogous to that obtained with SLN n.4. It is noteworthy that lecithin plays a significant role in insulin encapsulation efficiency, probably conferring a certain structural rigidity to the oil/water interface; moreover, the

proper modulation of W/O/W emulsion composition in term of inner aqueous phase and oil phase ratio, could probably positively influence insulin entrapment.

#### **4. Conclusions**

SLN obtained applying the solvent-removing technique to W/O/W emulsions containing partially water-miscible solvents allowed to encapsulate insulin, which was chosen as hydrophilic peptide drug. Main results worth underlining are the actual possibility to use non toxic solvents and lipidic biocompatible matrixes, such as monoglycerides, in the formulation of SLN, and the obtainment of nanoparticles with mean diameters below 1  $\mu\text{m}$ , allowing to hypothesize a possible *in vivo* application. On the other hand, owing to the small volume of the inner aqueous phase in a W/O/W system, and to a non-negligible permeability of both interfaces, great difficulties are still present in encapsulating high percentages of insulin, even if fair concentrations of the peptide are reached as a consequence of the acidic medium.

Further efforts are therefore necessary to improve the encapsulation efficiency in order to propose multiple emulsion-derived SLN for preliminary *in vivo* studies.

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Table I

Physicochemical and toxicity data of solvents employed in W/O/W emulsions formulation

Solvent	bp (°C)	water solubility 20°C (g/l)	ORL-RAT DL <sub>50</sub> (mg/kg)*
BL	190	77.0	>5000
EA	76	150.4	5620
IPA	88	30.9	6750
PA	101	18.9	9370
MC	40	13.4	1600

\*(Sigma Aldrich MSDS)

Table II

Solubility of GMS and CP in the solvents employed in W/O/W emulsions

Solvent	CP(mg/ml)		GMS (mg/ml)	
	25°C	50°C	25°C	50°C
BL	<50	250	<50	500
H <sub>2</sub> O-saturated BL	<50	250	<50	100
EA	<50	1300	<50	500
H <sub>2</sub> O-saturated EA	<50	1300	<50	250
IPA	60	1500	<50	800
H <sub>2</sub> O-saturated IPA	<50	1500	<50	500
PA	75	1550	<50	1000
H <sub>2</sub> O-saturated PA	55	1500	<50	500

Table III

## Solvent-solubility of insulin

Solvent	Insulin ( $\mu\text{g/ml}$ )	
	25°C	50°C
BL	insoluble	120
H <sub>2</sub> O-saturated BL	insoluble	95
EA	insoluble	40
H <sub>2</sub> O-saturated EA	insoluble	insoluble
IPA	insoluble	<20
H <sub>2</sub> O-saturated IPA	insoluble	30
PA	insoluble	<20
H <sub>2</sub> O-saturated PA	insoluble	

Table IV

Insulin apparent solvent/0.01 M HCl partition coefficients

Solvent	$P_{app}$
MC	$0.05 \pm 0.01$
EA	$0.12 \pm 0.01$
PA	$0.04 \pm 0.02$
IPA	$0.05 \pm 0.01$
BL	$0.06 \pm 0.02$

Table V

w/w percent compositions of W/O/W emulsions

Em	BL	EA	PA	GMS	CP	0.01 M HCl	2.0% P68	E200*	T80	S165**
1	58.2	/	/	1.0	0.5	14.9	24.8	/	0.6	/
2	/	58.2	/	1.0	0.5	14.9	24.8	/	0.6	/
3	/	/	58.2	1.0	0.5	14.9	24.8	/	0.6	/
4	23.1	/	/	2.4	/	3.5	70.8	0.3	/	/
5	/	23.1	/	2.4	/	3.5	70.8	0.3	/	/
6	/	/	23.1	2.4	/	3.5	70.8	0.3	/	/
7	42.8	/	/	4.0	/	8.7	44.1	0.2	/	0.2

\* Epikuron®200

\*\* Simulsol®165

Table VI

Mean diameters and encapsulation efficiencies of insulin-loaded SLN

SLN	mean particle size (nm)		polydispersion		encapsulation efficiency (%)	loading (mg insulin/g SLN)
1	1180*	1220**	0.205*	0.235**	10.1±1.6	7.98±1.26
2	2230	2150	0.257	0.274	6.4±2.3	5.08±1.82
3	2420	2380	0.174	0.199	5.2±2.2	4.13±1.74
4	670	690	0.206	0.196	40.3±2.5	12.02±0.75
5	874	918	0.215	0.205	23.9±1.2	7.13±0.36
6	790	775	0.263	0.251	18.8±3.0	5.61±0.89
7	594	612	0.094	0.117	25.7±4.5	12.27±2.14

\* just after preparation

\*\* re-dispersed after centrifugation at 24 000 rpm, washing and drying



Figure 1

Insulin stability in water at 50°C in the presence of 3.0% w/v solvents and 0.2% w/v surfactants

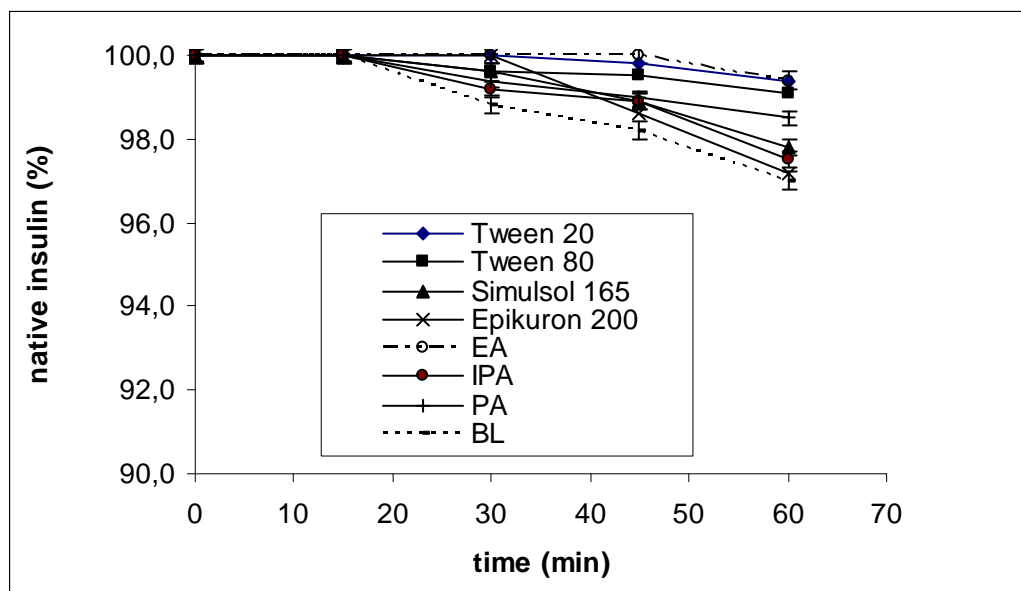


Figure 2

Insulin aggregation rate at different solvent/water interfaces (unstirred) plotted as Transmittance percentage as a function of time.

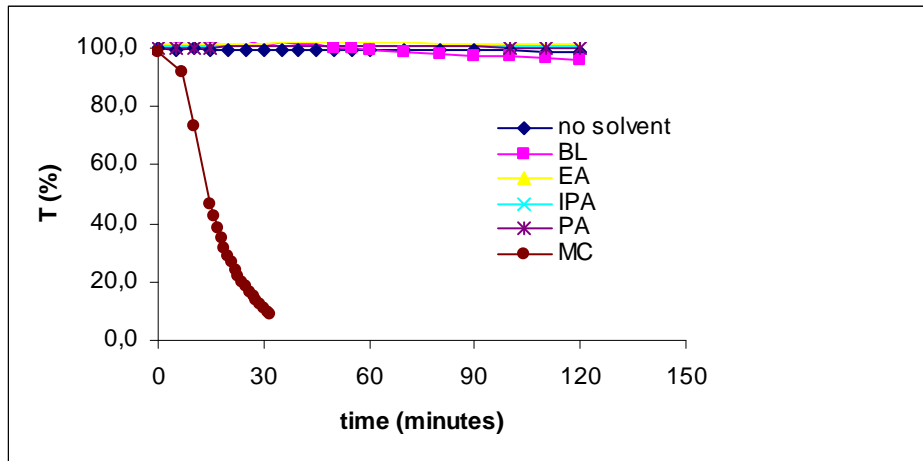
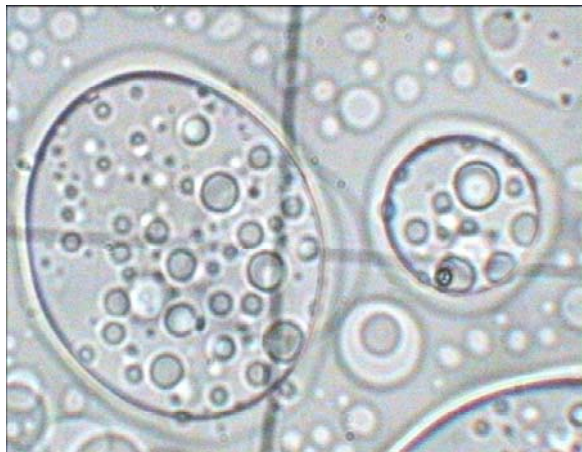


Figure 3

Micrographs of W/O/W emulsions

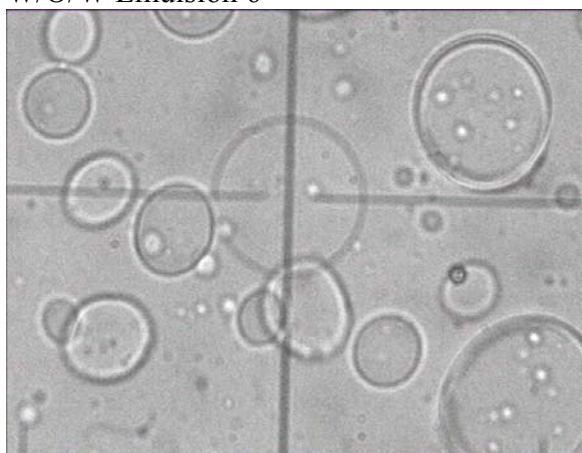
W/O/W Emulsion 1



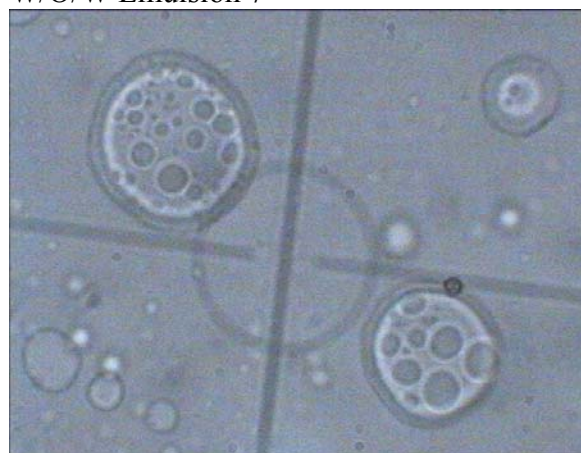
W/O/W Emulsion 4



W/O/W Emulsion 6



W/O/W Emulsion 7



— 1  $\mu$

Figure 4

Differential scanning calorimetry of GMS bulk material, GMS-Epikuron 200 SLN suspensions, and Epikuron liposomes suspension.

DSC

