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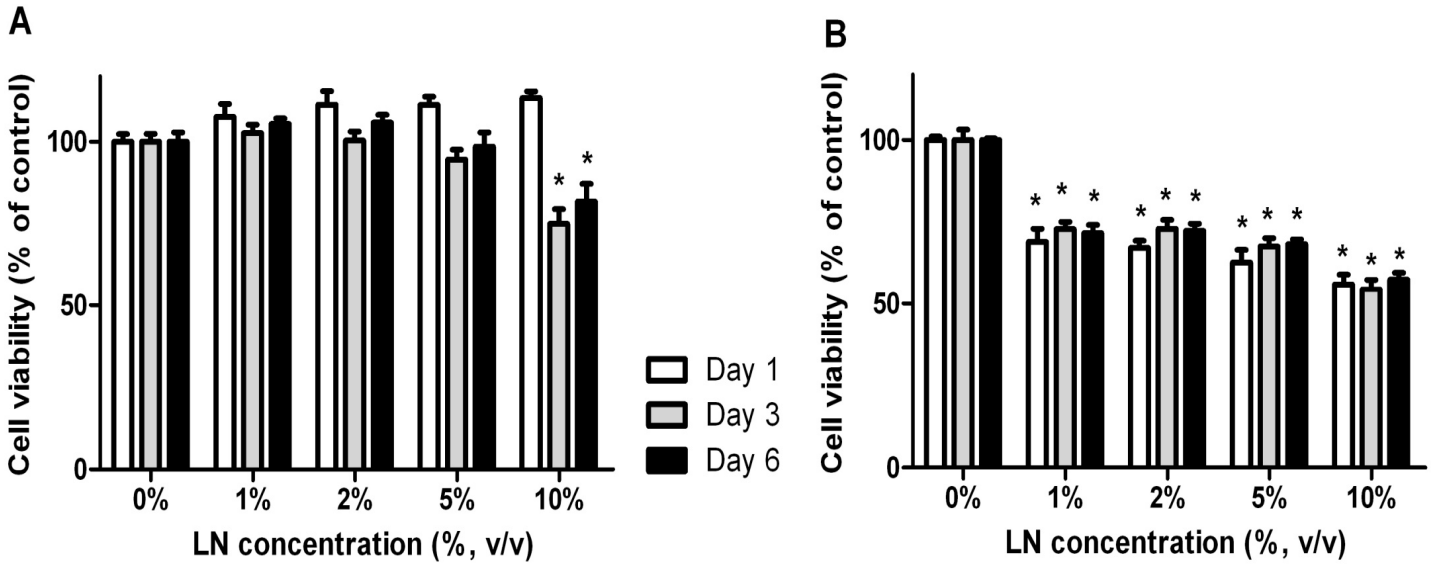

Cell viability of Caco-2 (A) and HepG2 (B) cells exposed to Softisan® 100 Lipid Nanoparticles (at 1%, 2%, 5% and 10%, v/v, diluted in FBS-free culture media) prepared by double emulsion technique. Results are expressed as % of control (untreated cells), and are average values (n=3) ± S.D.

Solid Lipid Nanoparticles for Hydrophilic Biotech Drugs: Optimization and Cell Viability Studies (Caco-2 & HEPG-2 cell lines)

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Abstract

Insulin was used as model protein to developed innovative **Solid** Lipid Nanoparticles (**S**LNs) for the delivery of hydrophlic biotech drugs, with potential use in medicinal chemistry. **SLNs** were prepared by double emulsion with **the purpose of promoting stability and enhancing the protein bioavailability.** Softisan®100 was selected as solid lipid matrix. The surfactants (Tween®80, Span®80 and Lipoid®S75) and insulin were chosen applying a 2^2 factorial design with triplicate of central point, evaluating the influence **of dependents variables as polydispersity index (PI), mean particle size (z-AVE), zeta potential (ZP) and encapsulation efficiency (EE) by factorial design using the ANOVA test. Therefore, thermodynamic stability, polymorphism and matrix crystalinity were checked by Differential Scanning Calorimetry (DSC) and Wide Angle X-ray Diffraction (WAXD), whereas the effect of toxicity of SLNs was check in HepG2 and Caco-2 cells.** Results showed a mean particle size (z-AVE) width between 294.6 nm and 627.0 nm, a PI in the range of 0.425 to 0.750, ZP about −3 mV, and the EE between 38.39% and 81.20%. After tempering the **bulk lipid (mimicking the end process of production), the** lipid showed amorphous characteristics, with a melting point **of** ca. 30ºC. The toxicity of **SLNs** was evaluated in two distinct cell lines (HEPG-2 and Caco-2), showing **to be dependent on the concentration of particles in HEPG-2 cells, while no toxicity in was reported in Caco-2 cells. SLNs were stable for 24 h in *in vitro* human serum albumin (HSA) solution. The resulting SLNs fabricated by double emulsion may provide a promising approach for administration of protein therapeutics and antigens.**

Keywords: Lipid nanoparticles, double emulsion, hydrophilic biotech drugs, insulin, Caco-2 cell lines, HEPG-2 cell lines

1. Introduction

It is know that peptides and proteins have low bioavailability through the oral route by denaturation in gastrointestinal fluids and low permeability in intestinal mucosa [1]. For this reason, the main route of administration used is parenteral, causing pain and discomfort to the patient. Among macromolecules, insulin is a peptide widely employed in the diabetes treatment. Insulin is a sensitive molecule susceptible to denaturation during the process of production, as well as in biological fluids and during storage. Nanotechnology applications are increasing in pharmaceutical drug delivery to solve most of stability and delivery problems with biotech drugs [2, 3]. Several studies have reported the loading of hydrophilic **biotech drugs in** Solid lipid nanoparticles **(SLNs) [[4-7].** SLNs have been used as drug carriers to prevent enzymatic degradation [8], to promote permeability across the cell membranes**, to improve** the bioavailability of hydrophilic drugs [9] and controlled release **strategies [10]. SLNs are of composed lipid matrix, stabilized by a surfactant. The advantageous of their lipid composition, include the physiological compatible compounds [11, 12], of generally recognized as safe (GRAS) status [13, 14], production without organic solvents and scale up to industrial production. SLNs can be produced by various traditional dispersion techniques as high pressure homogenization [15], double emulsion [16], microemulsion [17] and emulsification solvent-evaporation method [18].** In this work, **SLNs** were produced by double emulsion method **loading insulin as hydrophilic model drug. To** achieve an optimal formulation, a 2^2 factorial design with triplicate at the central point was applied. Selected independent variables were the ratio of surfactants and insulin concentration, whereas the dependent variables were the mean particle size (z-AVE), polydispersity index (PI), zeta potential (ZP) and encapsulation efficiency (EE). The study was completed with the evaluation of the thermodynamic stability, polymorphism and matrix crystalinity by Differential Scanning Calorimetry (DSC) and Wide Angle X-ray Diffraction (WAXD), whereas the effect of toxicity of **S**LNs was check in HepG2 and Caco-2 cells.

2. Material and methods

2.1. Material

Glycerides of hydrogenated coconut (Softisan® 100) were a gift from Sasol GmbH (Germany). Polysorbate 80 (Tween® 80) and Coomassie blue were obtained by Sigma (Portugal). Mono/oleate sorbitan oleate (Span® 80) was donated by Croda (Brazil), soya lecithin

hydrogenated (Lipoid® S75) were donated as a gift from Lipoid (Germany); insulin (Humalog® mix 25) was purchased from Libbs (São Paulo, Brazil). Penicillin, streptomycin, L-glutamine, hank´s balanced salt solution (HBSS) were purchased from Gibco (Alfagene, Portugal). Alamar blue® was obtained from Invitrogen (Alfagene, Portugal). Human albumin was obtained by Sigma Aldrich (Portugal). Double distilled water was used after filtration in a Millipore system (home supplied).

2.2. Methods

*2.2.1. **SLNs** preparation*

The double emulsion (w/o/w) method was chosen to avoid high temperatures that could produce protein denaturation or change its properties. Insulin was selected as the model drug. For the production of internal water phase (IWP) (insulin and water) and the lipid phase (LP) (500 mg Softisan®100, 5 mL glycerol, Span®80/ Lipoid®S75) were, separately, heated to a temperature 10 ºC above the lipid phase transition. The IP was added to LP and homogenized with high shear homogenization (Ultra-Turrax®, T25, IKA, German) for 10 minutes, intensity of 10 000 rpm at constant temperature. A part of external water phase (EWP) composed of 0.25 g Tween®80 and 40 mL water was cooled down to 3 ºC and added to previously formed emulsion (w/o). The multiple emulsion was maintained by high shear homogenization for 2 min at 10 000 rpm. Then, it was transferred to magnetic stirring (Tecnal, TE-0851, Brazil), and added to the other part of EP and kept under stirring for 20 min.

2.2.2. Factorial design

The influence of the surfactant composition and ratio on the double emulsion properties has been studied by determining the Hydrophilic Lipophilic Balance (HLB), as described by Schmidts et al., 2009 [19], and applying the following equation:

$$HLB = \frac{HLB(I) \times \frac{\phi\left(\frac{w}{o}\right)}{w} \times wt(\%)(I) + HLB(II) \times wt(\%)(II)}{\frac{\phi\left(\frac{w}{o}\right)}{w} \times wt(\%)(I) + wt(\%)(II)} \times 100$$

where HLB(I) is the HLB value of the internal emulsifier used (I) for the primary w/o emulsion, $\phi\left(\frac{w}{o}\right)\Big/w$ is the w/o fraction in the multiple emulsion, wt%(I) is the per cent by weight of emulsifier I in the w/o emulsion, HLB(II) is the HLB value of emulsifier II and wt%(II) is the per cent by weight of emulsifier II in the multiple emulsion [19]. The influence of the surfactant ratio Span®80/ Lipoid®S75 and the insulin concentration on the LNs was evaluated using a 2^2 factorial design with triplicate of central point for estimating the experimental error, composed of 2 variables which were set at 2-levels each. The mean particle size, PI, ZP and EE were the dependent variables. The design required a total of 7 experiments for each formulation. Each factor, the lower and higher values of the lower and upper levels, were represented by a (−1) and a (+1) and the central point was represents by (0) as summarized in Table 1. These were chosen on the basis of the tested lower and upper values for each variable according to pre-formulation studies and literature research. The data were analyzed using **ANOVA by** STATISTICA 7.0.

[Please, insert Table 1 near here]

2.2.3. Particle size, polydispersity index, and zeta potential analysis

The **SLNs** was evaluated with respect to the hydrodynamic mean size, PI, and ZP. The mean size was determined by Dynamic Light Scattering (DLS; Zetasizer Nano NS, Malvern, UK). The samples were diluted with ultra-purified water to weaken the opalescence before particle size measurements. ZP was analyzed in NaCl 0.9% (w/v) adjusting conductivity to 50 μS/cm. The ZP was calculated from the electrophoretic mobility using the Helmholtz–Smoluchowski equation [20]. The analysis was performed using the software included in the system.

2.2.4. Encapsulation efficiency (EE)

The protein encapsulated into **SLNs** was quantified by the Bradford method [21], using bovine serum albumin (BSA; Sigma, Portugal) as a standard. The absorbance reading was held in spectrophotometer at a wavelength of 595 nm, using a quartz cuvette. The calibration curve was performed with concentrations of 0, 10, 15, 20, 25, 30, 40, 50, 70, 90, 110, 140, 170, 200, 230, 270, 310, 350, 400, 400, 450, 500, 600 μg/mL. To determine the EE of **SLNs** samples were centrifuged (60 000 rpm during 30 minutes (ScanSpeed mini centrifuge from Scanlaf, Denmark)) and 500 μL of the supernatant was added to a solution of *Coomassie Brilliant blue* (Bradford reagent), followed by agitation. Then EE was calculated using the following equation:

$$EE\ (\%) = \frac{(Total\ of\ Protein\ in\ IWP) - (Total\ of\ Protein\ in\ Supernatant)}{Total\ of\ Protein\ in\ IWP} \times 100$$

2.2.5. Differential Scanning Calorimetry (DSC)

Thermal behavior of lipid matrices was assessed by DSC (Mettler Toledo, FP90 Central Processor, São Paulo, Brazil). A volume of sample containing approximately 5-10 mg of lipid mass was weighed in an aluminum pan and sealed hermetically, under inert atmosphere (N_2). The analysis was performed at a heating and cooling rate of 5 K/min, using an empty pan as reference. **The samples were heated up from 10 to 100°C, following cooling down to 10°C.**

2.2.6. Wide Angle X-ray Diffraction (WAXD)

To study the polymorphism and crystalline properties of **SLNs**, WAXD was carried out in a X-ray diffractometer (Philips, model X´pert, Pennsylvania, USA), using copper anode which delivered X-ray of wavelength, λ = 0.154056 nm. WAXD measurements were taken from 5° to 33° in 0.015° steps (1 s per step). The interlayer spacing was calculated from the reflections using the Bragg's equation $\eta\lambda = 2d\sin\theta$ where λ is the wavelength of the incident X-ray beam, *n* an integer and θ is the scattering angle. The parameter *d*, otherwise called the interlayer spacing, is the separation between a particular set of planes of the crystal lattice structure. Data of the scattered radiation were recorded with a blend local-sensible detector using an anode voltage of 40 kV, a current of 25 mA and a scan rate of 0.5º per minute. The samples were mounted on a thin glass capillary being fastened to a brass pin without any previous sample treatment.

2.2.7. Cytotoxicity assay using HepG-2 and Caco-2 cells

The biocompatibility of **SLNs** formulations could be assessed performing the Alamar blue® assay [22]. Alamar blue® (resazurin) is a sensitive oxidation-reduction indicator, after cells reduction it fluoresces and changes coloration. The reduction is mediated by mitochondrial enzymes of metabolically active cells [23, 24]. In this study, the two cell lines HepG2 (human hepatoma cell line; ATCC, Rockville, MD, USA) and Caco-2 (human colon adenocarcinoma; CLS, Eppelheim, Germany), were used, separately cultured in flasks. These cultures were maintained in culture medium, Dulbecco's Modified Eagle Media (DMEM), containing 25 mM glucose supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco, Life technologies), 2 mM

L-glutamine (Gibco, Life technologies) and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin, Life technologies) at 37°C in normal atmosphere of 5% CO_2 in air. The supernatant of confluent cells was removed and cells were exposed to trypsin until complete detachment and disaggregation (at 37°C for ~6 or 10 minutes, for HepG2 or Caco-2 cells, respectively). Trypsin reaction was stopped with culture medium, cells were ressuspended, counted (Neubauer chamber), diluted in culture media at a density of $5x10^4$ cells/mL, seeded onto 96-well microplates (100 µL per well; $5x10^3$ cells/well) and cultured for 24 hours. After that, culture media was removed and replaced by FBS-free culture media supplemented with SLNs formulation (final SLNs concentration 1%, 2%, 5% and 10% (v/v)), and incubated for ana additional 24 h. For estimation of the cell survival rate, 10% (v/v) Alamar Blue (Invitrogen Corporation) was added to the medium, and absorbance was monitored (Multiskan EX, Labsystems) at wavelengths 570 nm and 620 nm after 4 h culture, as described by the manufacturer's protocol, and at several timescale points as indicated in the results section. The percentage of Alamar Blue reduction was calculated using the equations recommended by the manufacturer's protocol using Excel tools.

2.2.8. In vitro stability

Dynamic light scattering (DLS) experiments were performed using an ALV-setup with an ALV-6000 correlator (ALV GmbH, Langen, Germany) in the cross-correlation mode detector and a 30 mW He-Ne laser with wavelength λ = 632 nm. The correlation functions were analyzed by numerical inverse Laplace transformation using the REPES program [25], which calculates the distribution of relaxation times, A(τ), from the measured intensity autocorrelation function, $g^2(t)$. From the centers of gravity of the peaks in A(τ), the diffusion coefficient D is obtained using $D = \Gamma/q^2$ where Γ is the relaxation rate ($\Gamma = \tau^{-1}$) and q the modulus of the scattering vector at angle θ, given by $q=4\pi n\sin(\theta/2)/\lambda$, with n the refractive index of the sample and λ the wavelength of the laser light in vacuum. The hydrodynamic radius, R_H, is calculated [26, 27] using the Stokes-Einstein equation: $R_H = k_B T/6\pi\eta D$, where k_B is Boltzmann's constant, T the absolute temperature, and η the temperature-dependent viscosity of water included in the REPES program used for analysis. The stability of the **SLNs** was monitored after placing them in contact with human serum albumin (HAS; Sigma, St. Louis, MO) solution. Typically, 6.25 µg/mL of the LNs was placed in contact with the model blood plasma protein HSA at concentration of 45 mg/mL in PBS. The dynamic light scattering experiments were performed periodically up to 24

h in order to monitor the stability of the system (especially, the mean size and size distribution of the LNs).

3. Results and discussion

The mean particle size of colloidal nanosized carriers usually range between 10 and 1000 nm. SLNs are receiving attention from researchers worldwide since they depict properties as small size and high drug payload. Therefore, SLN have been tested by various administration routes as parenteral, oral and topical. The composition is based on a lipid matrix in an aqueous surfactant solution. SLNs produced by double emulsion are able of loading hydrophilic drugs in internal aqueous phase.

In this work, insulin was used as protein model due to its low cost and commercial availability; as it is expected to mimic the loading of other therapeutic peptides, small proteins, and nucleic acids.

To optimize the formulation, seven different experiments were carried out varying the surfactant ratio and the insulin content of the formulation, and the obtained z-AVE, ZP, PI and EE were measured. Results are shown in **Figure 1**, arranged according the factorial design. The z-AVE width ranged between 294.6 nm and 627.0, PI between 0.425 and 0.750 and ZP obtained was about −3 mV, and the EE varied between 38.39% and 81.20%.

Particle size is important to the evaluation of the stability of colloidal systems. The particles are **between 10 and 1000 nm, the size is influence by the surfactant and by the homogenization technique. Using high shear homogenization is expecting particle with size and PDI size. To overcome, it is possible increase time of homogenization or adding a charged surfactant.**

We aimed **to evaluate** the influence of the surfactant ratio (Span® 80/ Lipoid® S75), the amount of insulin and the interaction between surfactant ratio and the amount of insulin on SLNs size, PdI, ZP and EE. Figure 1 represents the Pareto charts where we observe that surfactant ratio (1) and the interaction between surfactant ratio and insulin amount (1by2) were significant in relation to particle size (Figure 1A). However, the surfactant ratio, the amount of insulin or the interaction (1by2) did not affect significantly the PI index (Figure 1B) and the ZP (Figure 1C). Figure 1D shows that the amount of insulin had a significant effect on EE. **The statistical analysis was carried out using ANOVA, and the proposed model was realized by each variable effect and interactions (insulin and surfactant), and the linear models obtained were the following:**

Size (nm) = 401.143 -175.8 surfactant ratio −154.6 interaction surfactant insulin

EE (%) = 62.30429 + 39.58500 insulin

[Please, insert Figure 1 near here]

Figure 2 shows the surface response charts of experimental design performed to study the influence of insulin amount and surfactant ratio on the **SLNs** size, PI, ZP and EE, respectively on Figure 2A, B, C and D. Figure 1A shows that when using lower surfactant ratio, higher z-AVE values were obtained for SLNs. The same results were reported for the PI values (Figure 2B). ZP (Figure 2C) showed little variation (–3.5 mV to –3.7 mV), as expected since both lipid and surfactant molecules are of non-ionic character. On the other hand, EE showed large variation from 38.39% to 81.20%. The EE was dependent on the amount of insulin added to the formulation. The surfactant concentration and surfactant ratio did not influence in EE (Figure 2D).

[Please, insert Figure 2 near here]

Figure 3A shows the thermogram result obtained for the empty **SLNs**. In the heating curve (upper trace) an endothermic peak is observable with onset temperature at 26.7°C, peaking at 30.4°C, extending to 36.6°C, temperature offset, the curve integration gave 0.78 J/g. Figure 3B represents the thermogram of insulin-loaded **SLNs**. Endotherm started at 25.6°C, with an enthalpy of 0.89 J/g. This effect depicted two peaks, one at 30.1°C followed by a second at 32°C. During the cooling phase, no peaks were recorded in the curve (data not shown), demonstrating no protein denaturation.

[Please, insert Figure 3 near here]

Lipids crystallize in two or three different phases, α and β´ or α, β and β´ [28, 29]. WAXD analyses of Softisan® 100 after and before tempering showed interference maxima in two peaks 20.66 (2θ) and 23.33 (2θ).

[Please, insert Figure 4 near here]

[Please, insert Figure 5 near here]

Tests of cell viability were carried out on Caco-2 cells and HepG2 cells exposed to empty **SLNs** formulated with 11.90 mg lipid/mL water and diluted in FBS-free culture media to the final concentrations of 1, 2, 5 and 10% (v/v). Cell viability was assayed with the Alamar blue® indicator, a non-toxic indicator that upon reduction by metabolically active cells changes color from blue to pink., and expressed as % of control (untreated cells). Figure 6A shows that Caco-2 cells can tolerate very well this formulation as no toxicity was observed for up to 5% (v/v) **SLNs** along the experiment. When Caco-2 cells are exposed to 10% **SLNs**, for 3 or 6 days, however we can observe a reduction in cell viability of about 20% ($p<0.5$), but 24 h of exposure to 10% **SLNs** does not affect cell viability. On the other hand, HepG2 (Figure 6B) cells exposed to SLNs showed a reduction on cell viability that was concentration dependent, we observe about 30% reduction just with 1% **SLNs** and about 40-45% reduction for 10% **SLNs**. Results allow stating that this formulation is not toxic in Caco-2 cells, whereas it shows some toxicity in HepG2 cells. **The low toxicity of these negatively charged SLNs observed in Caco-2, comparing to HepG2 has been attributed to a different degree of expression of several multidrug resistance proteins (ABC transporters). It has been shown that the Caco-2 express different types of ABC transporters [30] that might mediate the efflux of these particles. Indeed Bhattacharjee and co-workers have recently shown that polymeric nanoparticles interacted with Caco-2 cells ABC transporters mediating resulting in different degree of efflux depended on the particle charge [31]. HepG2 cells are also equipped with some ABC transporters which could be less efficient in mediating the SLN efflux, and thus HepG2 would be more susceptible to their presence. Differences on cell toxicity, depending on cell type is not surprising as we have recently reported that SLN induced-cytotoxicity is dependent on several factors, including cell type and SLN composition [32].**

[Please, insert Figure 6 near here]

The serum albumins are of particular interest for their ability to anchor both hydrophilic and hydrophobic surfaces and participate in a cascade of protein adhesion at surfaces [33]. Therefore, **SLNs** stability in the serum is a criterion for their usefulness as drug carrier *in vivo*. The stability of the **SLNs** was monitored when in contact with the model protein HSA in PBS (up to 24 h). The experiments were performed in the presence of 45 mg/mL of HSA which is nearly the amount found in the blood plasma. These experiments were performed by monitoring the system in contact with dynamic light scattering over time. The DLS results are given in Figure 7, and demonstrate that **SLNs** are stable for up to 24 h in the presence of HSA since no particles

aggregates where detected. However, regardless the **SLNs** stability during the experiments, the hydrodynamic radius (R_h) increases from 86 nm to 105 nm in 24 h (Figure 8) clearly demonstrating the increase in HSA adsorption in the **SLNs** surface along the time. Since **SLNs** surface consists of the hydrophilic polysorbate (Tween®80) and previously experiments with human plasma showed protein folding process in this surfaces the HSA adsorption cannot be avoided [34]. Finally, in the Figure 7 one may notice the development of a small amount of large aggregates after 24 h. This was experimentally evidenced to be related to the aggregation of protein molecules themselves rather than the **SLNs** since the same behavior was observed in SLNs used as control without HSA solutions.

[Please, insert Figure 7 about here]

[Please, insert Figure 8 about here]

4. Conclusions

This research investigated the influence of surfactant ratio and insulin EE. The method of production is easy and demands low costs. Size, PI and ZP are properties associated with the stability and acceptability of formulation. The optimized **SLNs** showed size, PI, ZP and EE around 300 nm, 0.5, −3 mV and 80%, respectively. The lipid showed an amorphous characteristic after melting. The toxicity was dependent on the cell type, showing concentration dependence to the HepG2, in which toxicity attained 45% reduction on cell viability for 10% **SLNs** and 6 days of exposure. But, only a slight toxicity was found for Caco-2 cells, for higher concentrations and for the longer periods of incubation. **SLNs** are stable for up to 24 h in the presence of HSA since no **SLNs** aggregates where detected and presented spherical morphology. This carrier show interesting properties for delivering hydrophilic biotech drugs.

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Declaration of interest

The authors report no conflicts of interest.

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Table Captions

Table 1. Factorial design 2^2 with triplicate of central point

.

Table 1.

Variables	Level		
	(–1)	**(0)**	**(+1)**
Span® 80/ Lipoid® S75	1:1	2:1	3:1
Insulin (mg)	1.75	3.50	5.25

Figure Captions

Figure 1. Pareto chart showing the influence of the surfactant ratio (1) insulin amount (2) and the interaction of both (1by2) on the SLNs size (A), polydispersity index (B), zeta potential (C) and encapsulation efficiency (D).

Figure 2. Surface response charts of experimental design of SLNs. Influence of insulin amount and surfactant ratio on size (A), polydispersity index (B), zeta potential (C) and encapsulation efficiency (D).

Figure 3. DSC thermogram of empty SLNs (A) and insulin-loaded SLNs (B).

Figure 4. Wide-angle X-ray diffractogram (WAXD) of Softisan® 100 before (dashed line) and after (solid line) tempering.

Figure 5. Wide-angle X-ray diffractogram (WAXD) of SLNs.

Figure 6. Cell viability of Caco-2 (A) and HepG-2 (B) cells exposed to empty SLNs formulations (at 1%, 2%, 5% and 10%, v/v, diluted in FBS-free culture media) prepared by double emulsion technique. Results are expressed as % of control (untreated cells), and are average values (n=3) ± S.D.

Figure 7. Distribution of the hydrodynamic radius (R_h) for SLNs (6.25 µg/mL) in the presence of 45 mg/mL HSA dissolved in PBS at various times during 24 h.

Figure 8. R_h vs. time of the LNs in presence of 45 mg/mL HSA dissolved in PBS.

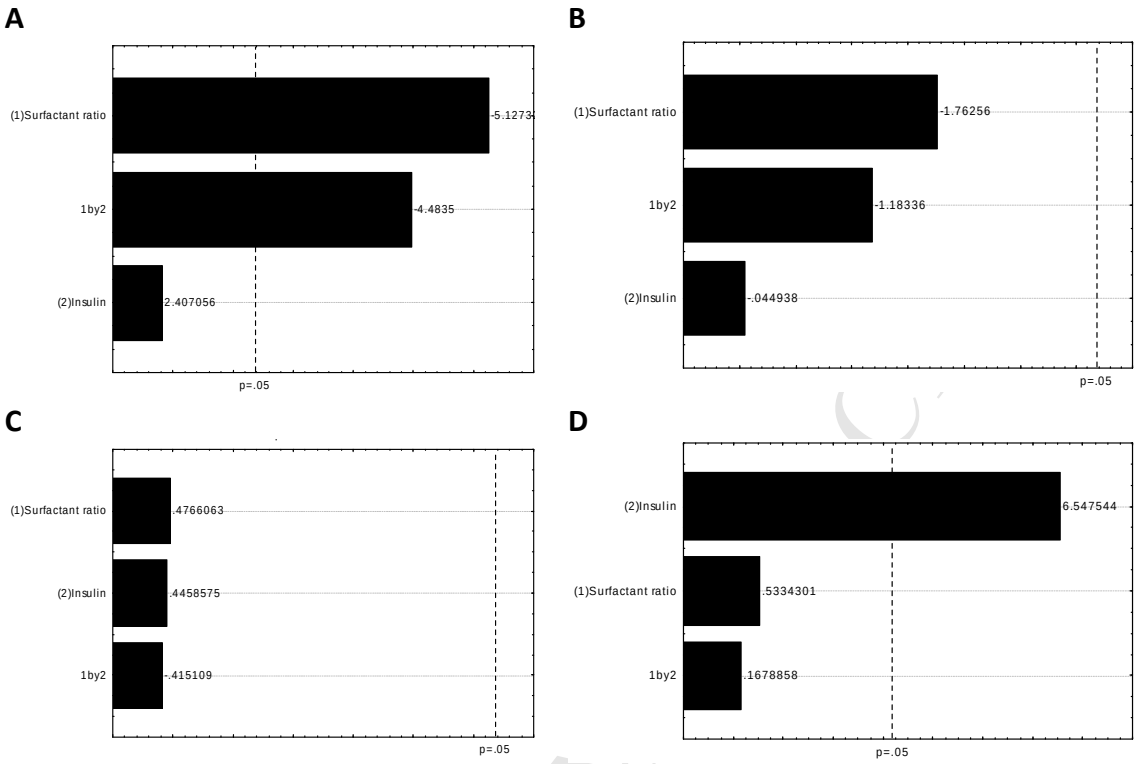

Figure 1

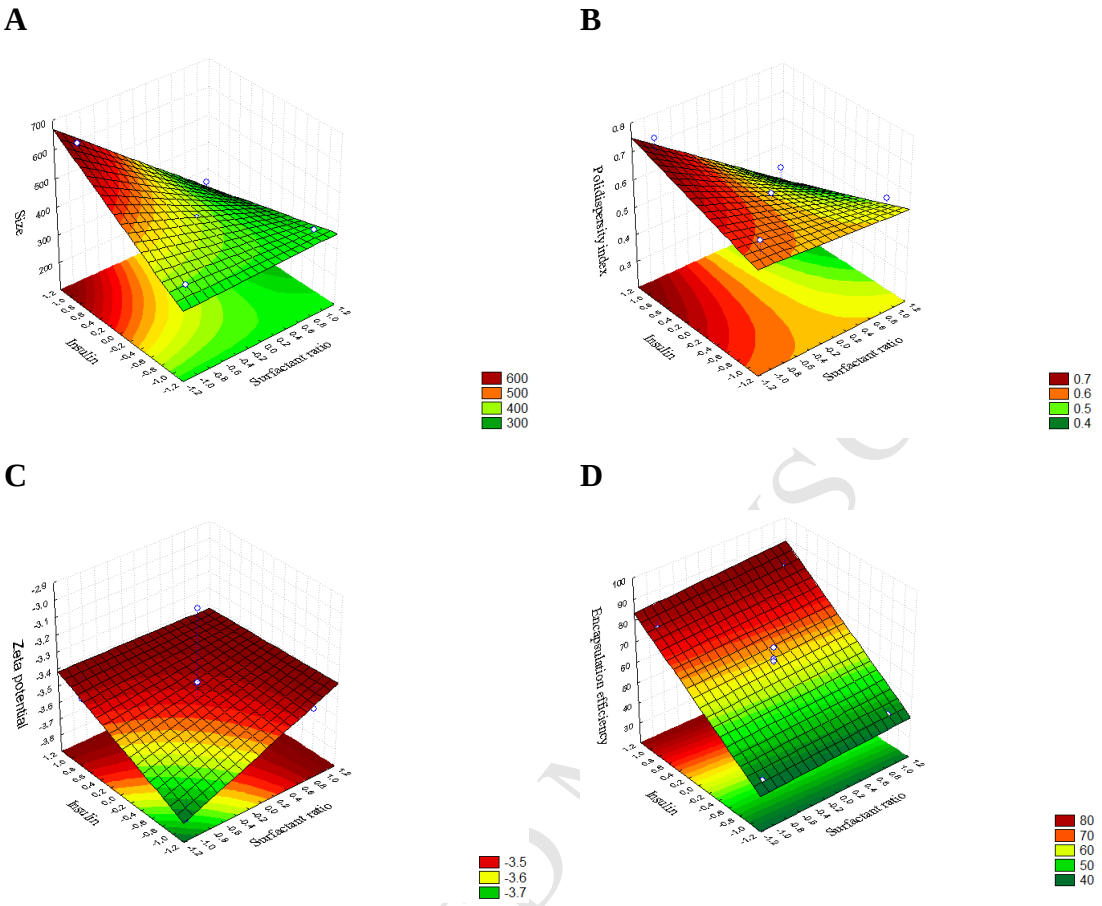

Figure 2

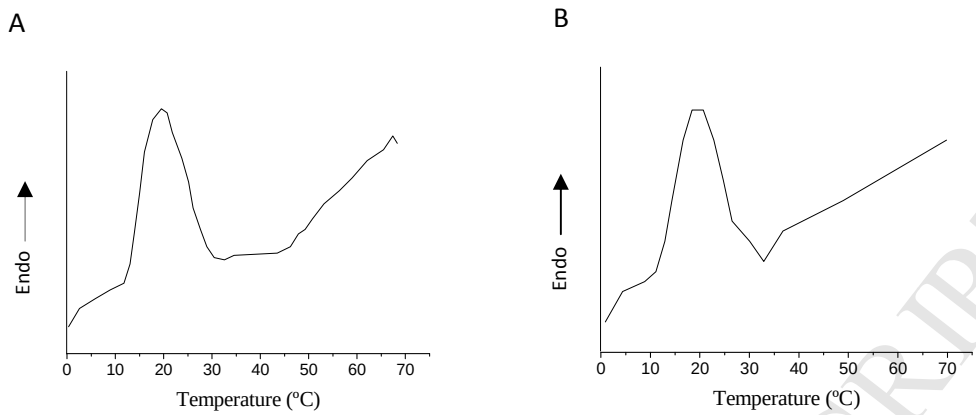

Figura 3

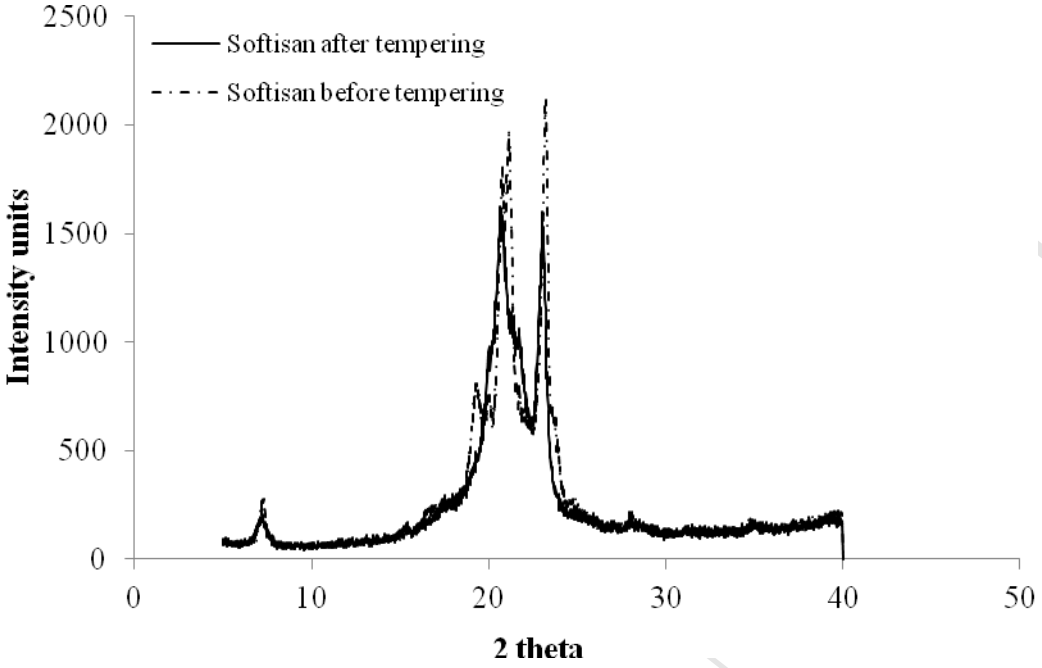

Figure 4

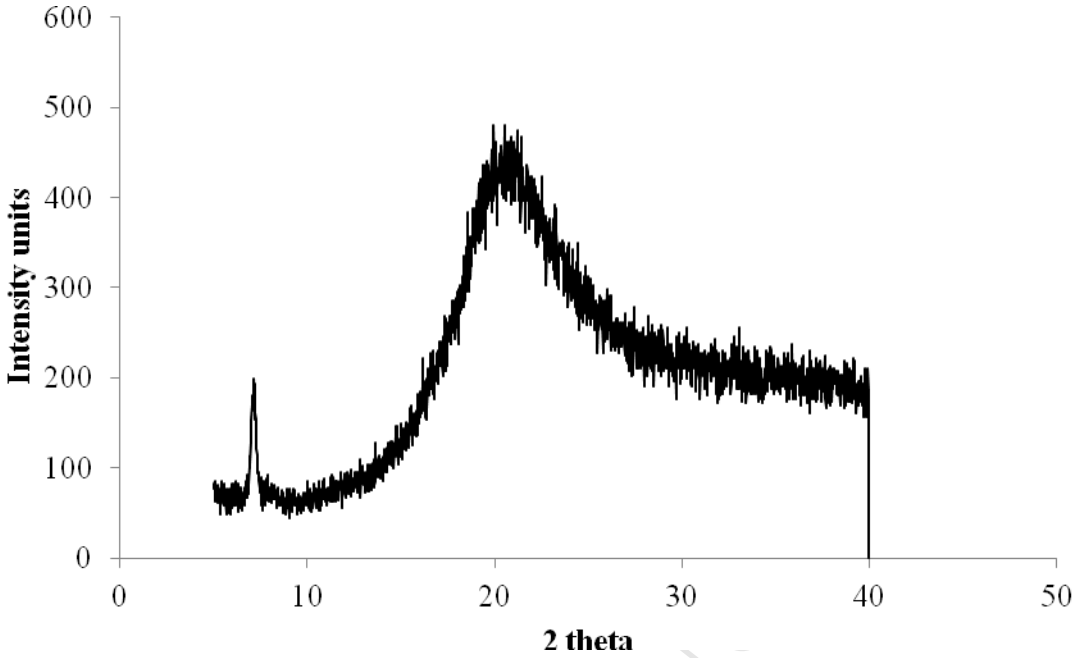

Figure 5

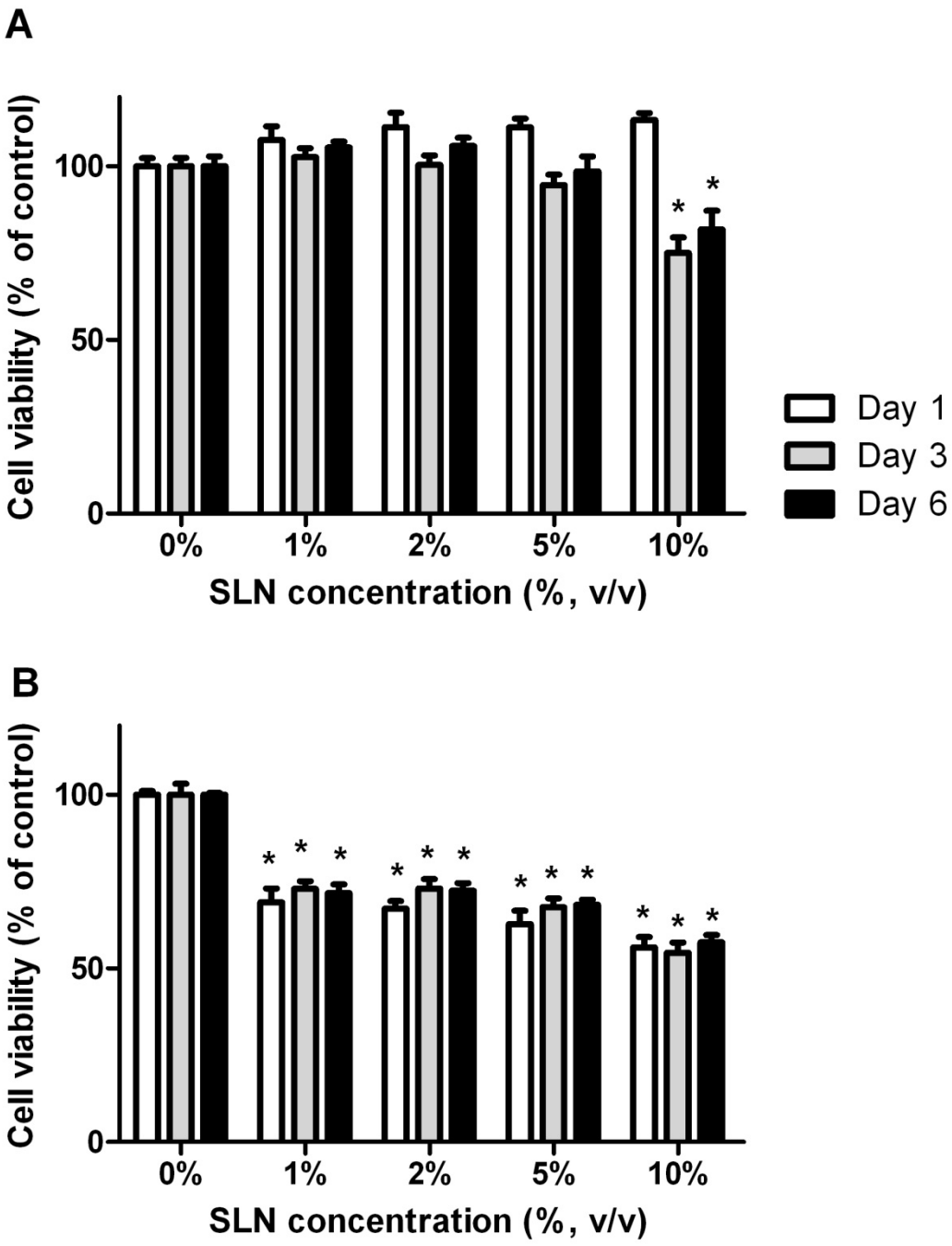
A
Cell viability (% of control)
100
50
0
0%
1%
2%
5%
10%
SLN concentration (%, v/v)
Day 1
Day 3
Day 6
B
Cell viability (% of control)
100
50
0
0%
1%
2%
5%
10%
SLN concentration (%, v/v)

Figure 6

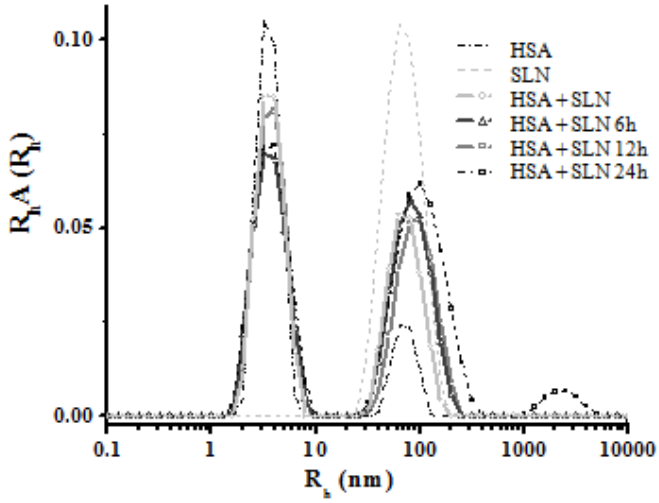

Figure 7

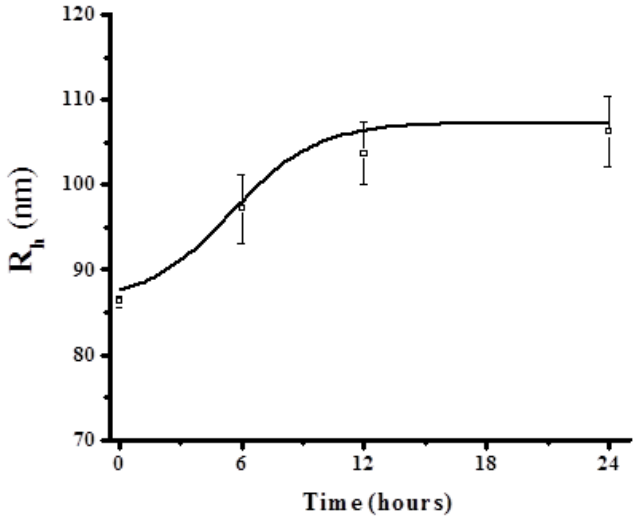

Figure 8

Research Highlights

- Softisan®100 lipid nanoparticles (LNs) were assembled applying a 2^2 factorial design.
- Toxicity in HEPG-2 and Caco-2 cells depends on LNs concentration.
- LNs are stable for 24 h in *in vitro* human serum albumin (HSA) solution.