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Research Article

# Optimization of nimesulide-loaded solid lipid nanoparticles (SLN) by factorial design, release profile and cytotoxicity in human Colon adenocarcinoma cell line

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**Abstract**

The aim of this work has been the development of a non-toxic, long-term stable solid lipid nanoparticles (SLN) formulation for the loading of Nimsulide (NiM) by a $2^2$ factorial design. The optimized formulation was composed of 10 wt% of glyceryl behenate and 2.5 wt% of poloxamer 188. Immediately after production, Z-Ave of NiM-SLN was 166.1±0.114 nm, with a polydispersity index (PI) of 0.171±0051 and zeta potential nearly neutral (-3.10±0.166 mV). A slight increase of Z-Ave was recorded for NiM-SLN stored at 25ºC for a period of 15 days, whereas at 4ºC particles kept size within similar range. Long-term stability was monitored using TurbiscanLab®, showing a high stability of the nanoparticles with variations in the backscattering profiles below 10%. The release profile of NiM-SLN followed a sustained pattern with ca. 30% of drug released up to 24 h. Empty-SLN and NiM-SLN were non-toxic after exposing Caco-2 cells to the highest concentration (100 μg/mL) up to 48 hours (cell viability higher than 80%). NiM-SLN were lyophilized using different cryoprotectants, producing particles of 463.1±36.63 nm (PI 0.491±0.027) with 5% trehalose. Solid character of NiM-SLN was confirmed by DSC, recording a recrystallization index of 83% for NiM-SLN and of 74% for lyophilized SLN.

**Keywords:** Nimesulide; Solid Lipid Nanoparticles; Physical Stability; Factorial Design Experiment; Lyophilization; Trehalose; Cryoprotectants

## 1. Introduction

Nimesulide (4-nitro-2-phenoxymethanesulfonamide) (NiM) is a non-steroidal anti-inflammatory drug (NSAID), widely used to ameliorate acute pain [1, 2]. Its mechanism of action is associated with the inhibition of the ciclo-oxigenase-2 (COX-2) isoform [3]. Like most of the NSAIDs, NiM shows low solubility in water, which limits its bioavailability *in vivo* [2]. This may lead to a significant reduction of drug's efficacy which, associated to the need of higher dose and/or long-term therapy, adverse side effects usually occur [1, 4].

One of the strategies to overcome solubility problems is the loading of the drug into nanoparticles. Solid lipid nanoparticles (SLN) have demonstrated potential to increase drug solubility and thereby its bioavailability, attributed to their lipid composition. Indeed, SLN are composed of biocompatible, non-toxic lipids, stabilized in aqueous media by suitable surfactants [5]. Surfactants are adsorbed onto the surface of the lipid cores during the production of SLN,

contributing to their long-term stability. The selection of the solid lipid for the production of NiM-loaded SLN (NiM-SLN) was performed via solubility screening studies based on the Hansen Solubility Parameters (HSP) [6]. The HSP parameters are composed by the dispersion forces, permanent dipole - permanent dipole and hydrogen bonding, and allow to compare the affinity of two materials and to find the best solvent. This evaluation is carried out through a HSP space (which is composed by suitable solvents) obtained experimentally and computationally by interaction studies between tested materials, and materials with well-defined HSP [7].

In this context, the novelty of this work was the optimization of NiM-loaded SLN by means of a full factorial design. The selected independent variables, namely the concentration of solid lipid and the surfactant, were evaluated for their influence on the dependent variables, namely the mean particle size (Z-Ave), polydispersity index (PI) and zeta potential (ZP) of the obtained SLN dispersions. The optimized formulation was selected from the factorial design experiment and physicochemically characterized by Turbiscan and DSC, following the selection of the best cryoprotector for freeze-drying the formulations. The *in vitro* release of the loaded NiM was evaluated in pH simulated media whereas cytotoxicity was checked in Caco-2 cell line.

## 2. Material and Methods

### 2.1. Materials

Nimesulide (NiM), phosphate buffer (pH 6.8), trehalose, sorbitol and mannitol were purchased from Sigma-Aldrich (Sintra, Portugal) and glycerol behenate (Compritol 888ATO) was a free sample from Gattefossé (Witten, France). Poloxamer 188 (P188) was a free sample from BASF (Ludwigshafen, Germany). Ultra-purified water was obtained from a Milli-Q Plus system (Millipore, Germany). All reagents were used without further treatment.

### 2.2. Factorial Design Experiment

A $2^2$ factorial design experiment based on two factors, with two levels each, was used to maximize the experimental efficiency, requiring a minimum of experiments. To this end, two different variables and their effect on the physicochemical properties of the obtained SLN were analyzed. The design involved a total of 7 experiments. The independent variables were the concentration of solid lipid glyceryl behenate, and the concentration of hydrophilic surfactant poloxamer 188. The selected dependent variables were the Z-Ave, PI and ZP. For each factor,

the lower, medium and higher values of the lower, medium and upper levels were represented by a (−1), a (0) and a (+1) sign, respectively, which are depicted in Table 1. Data curation was carried out using the STATISTICA 7.0 (Stafsoft, Inc.) software.

**[Please insert Table 1 about here]**

**Table 1.** Independent variables and levels used in the factorial design.

### 2.3. Production of SLN

SLN were produced by the hot high pressure homogenization (HPH) method. The solid lipid was heated 5-10 °C above its melting point, and it was subsequently added to a solution of surfactant, previously heated at the same temperature. A pre-emulsion was obtained under stirring with the Ultra-Turrax (Ultra-Turrax ®, T25, IKA), at 8000 rpm for 30 s. This pre-emulsion was further passed through a two-stage high pressure homogenizer (EmulsiFlex®-C3, Avestin), during 5 min, applying respectively, 600 and 60 bar, in the first and second stages. The homogenizer was previously heated at 85 ± 0.5 ºC, by recirculation with hot Milli-Q water. During the homogenization process, this temperature was maintained to guarantee that the lipid did not solidify. The o/w nanoemulsion formed was transferred to glass vials and immediately cooled down to room temperature to generate SLN. For NiM-loaded SLN, the drug was added to the solid lipid before melting and homogenization. The drug was used in a concentration of 5 µg/mL.

### 2.4. Particle size and zeta potential analysis

The particle size parameters Z-Ave and PI were determined by dynamic light scattering and ZP was determined by electrophoretic light scattering using the same equipment (Zetasizer Nano ZS, Malvern Instruments, Malvern UK). All samples were previously diluted with ultra-purified water to suitable concentration and analysed in triplicate. For the determination of ZP, ultra-purified water with conductivity adjusted to 50 µS/cm was used.

### 2.5. Stability analysis by TurbiscanLab®

The physical stability of SLN formulations was assayed by the TurbiscanLab® (Formulaction, France). SLN formulations were placed in a cylindrical glass cell and were evaluated at room

temperature (25 ± 2.0 °C) and at 4 ± 2.0 °C. This optical analyser is composed of a near-infrared light source (λ = 880 nm), and 2 synchronous transmission (T) and backscattering (BS) detectors. The T detector receives the light which crosses the sample, whereas the BS detector receives the light scattered backwards by the sample. The detection head scans the entire height of the sample cell (20 mm longitude), acquiring T and BS, each 40 µm, for 3 times during 10 min at different analyzed time points (0, 7 and 15 days after production).

### 2.6. Differential scanning calorimetry (DSC)

The crystallinity profile of SLN produced was assessed by DSC. A volume of SLN corresponding to 1–2 mg of lipid was scanned using a Mettler DSC 823e System (Mettler Toledo, Spain). Heating and cooling runs were performed from 25 °C to 90 °C and back to 25 °C at a heating rate of 5 °C/min, in sealed 40 µL aluminum pans. An empty pan was used as a reference. Indium (purity > 99.95%; Fluka, Buchs, Switzerland) was employed for calibration purposes. DSC thermograms were recorded and DSC parameters including onset, melting point and enthalpy were evaluated using STAR$^{e}$Software (Mettler Toledo, Switzerland). The recrystallization index (RI) was determined using the following equation [8]:

$$RI\ (\%) = \frac{\Delta H_{SLN\ or\ NLC\ aqueous\ dispersion}}{\Delta H_{bulk\ material} \quad x \quad Concentrat \quad ion_{lipid\ phase}} x\ 100$$

where ΔH is the molar melting enthalpy given by J/g and the concentration is given by the percentage of lipid phase.

### 2.7. Freeze-drying

Freeze-drying was performed in a Gamma 2-20: A Gamma 2-20 freeze dryer (Christ, Osterodea H., Germany) operating at 25 ºC/0.025 mBar during 24 h (main freezing) and during 6 h of drying. NiM-SLN were diluted 1:1 or 1:2 (v/v) with aqueous surfactant solution before freeze-drying, and frozen down to -80 ºC (Gamma 2-20) in a freezer immediately before freeze drying. NiM-SLN diluted in 1:1 or 1:2 ratios with double distilled water were used in each freeze-drying run as a control without cryoprotectant. For the storage of freeze-dried NiM-SLN, particles were kept in sealed glass vials (15 mL) at room temperature (25ºC) and under light protection.

### 2.8. Determination of Encapsulation Efficiency and Loading Capacity

The quantification of nimesulide in the optimized SLN formulation has been carried out by High Performance Liquid Chromatography (HPLC) method, using a 50 mm × 4.6 mm i.d. monolithic column (Chromolith®, Merck). The mobile phase consisted of acetonitrile-phosphate buffer (pH 7.0; 10 mM) (34:66, v/v), applying a flow rate of 4.0 ml $min^{-1}$. The detection wavelength was 255 nm, exhibiting linearity applying the equation $Y=0.0369X-7x10^{-5}$ ($R^2=0.9994$). Non-encapsulated drug was removed by centrifugation (60,000 rpm for 2 hours at 4 ºC). The amount of nimesulide in the supernatant was quantified after appropriate dilution, and the encapsulation efficiency (EE) was determined using the following equation:

$$\%\boldsymbol{EE} = \left(\frac{[\textbf{Total Weight of Nimesulide (g) - Nimesulide in Supernatant (g)}\ ]}{\textbf{Total Weight of Nimesulide (g)}}\right) \times 100$$

### 2.9. *In vitro* release studies

The release of NiM from SLN was performed using the dialysis bag diffusion method. The dialysis bag retains SLN and allows the released NiM to diffuse into dissolution media. The bags were soaked in Milli-Q water for 12 h before use. Ten milliliters of SLN were poured into the dialysis bag and the two ends of the bag were sealed with clamps. The bags were then placed in a vessel containing 80 mL phosphate buffer (pH 6.8), the receiving phase. The vessel was placed in a thermostatic shaker (New Brunswick, USA), at 25 ºC, with a stirring rate of 150 rpm. At predetermined time intervals, 1 mL samples were withdrawn, and analyzed by determining the absorbance using a Specord 250 Plus (Analytik Jena AG, Germany) at 555 nm. All samples were analyzed in triplicate. The percentage of NiM released was against a calibration curve of drug ($Y=0.0369X-7x10^{-5}$, and $R^2=0.9994$).

### 2.10. Cytotoxicity in Caco-2 cell line

Cytotoxicity of NiM-SLN was studied in a Caco-2 cell line purchased from Cell Lines Services (CLS, Eppelheim, Germany). Cells were kept in Dulbecco's Modified Eagle Medium (DMEM), containing 25 mM glucose supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco, Life technologies), 2 mM L-glutamine, and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin), at 37 ºC in an air with controlled humidity atmosphere of 5% $CO_2$ [9]. Cells were treated, in a time- and concentration-dependent manner, with NiM-free or loaded-SLN at different concentrations (12.5, 25, 50 and 100 µg/mL), produced in aseptic laminar flow

chamber. The supernatant of confluent cells was removed and cells were exposed at 37°C for 10 minutes to trypsin until complete detachment and disaggregation. Trypsin reaction was terminated with culture medium, cells were then re-suspended, counted (Neubauer chamber), seeded into 96-well microplates at a density of $5 \times 10^4$ cells/mL (100 µL/well), and cultured for 24 hours. Culture media was then removed and replaced by FBS-free culture media supplemented with SLN formulation (final SLN concentration 1%, 2%, 5% and 10% (v/v)), and incubated for 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 after 24 and 49 hours. Results were analysed by determining the percentage of AB reduction, expressed as percentage of control (untreated cells). Statistical analysis was performed by Student's *t*-test ($p < 0.05$) [10].

### 2.11. Statistics

Statistical analysis of data was performed using STATISTICA 7.0 (Stafsoft, Inc.) software, being a p-value < 0.05 accepted as significant. Data were expressed as the mean value ± standard deviation (Mean ± SD), usually from three independent experiments (n = 3).

## 3. Results and Discussion

Pre-formulation studies, by screening the solubility of the drug in the solid lipid, are a key step to enable the development of an optimized SLN formulation [6], in order to achieve a high EE (99.6%). The type of surfactant and solid lipid used must ensure chemical compatibility in order to stabilize the systems and improve their biocompatibility [5]. Glyceryl behenate and Poloxamer 188 were selected as solid lipid and surfactant, respectively. Poloxamer 188 is an hydrophilic emulsifier with a HLB value of close to 22 [11].

Physicochemical characteristics and stability of the new drug delivery systems are crucial issues to be addressed during the formulation stage, entailing for their careful monitoring during processing. The initial formulation consisted of 10 wt% of solid lipid and 2.5 wt% of surfactant. The obtained physicochemical parameters of this initial formulation summarized in Table 2 translate the successful choice of solid lipid and surfactant combination.

A second evaluation was carried out with the aim to analyse the influence of the concentration of both components on the dependent variables (Z-Ave, PI and ZP). The effect of the solid lipid concentration on the quality of the final dispersion is substantially important, since the increase of the lipid content is always related to increased drug payload [12, 13]. With this in mind, a $2^2$ full factorial design was applied for the development of an optimized SLN dispersion [14]. The impact of the independent variables was assessed upon the selected dependent variables, namely the Z-Ave, PI and ZP (Table 1). The Z-Ave of the 7 assayed formulations is shown in Table 3, ranging between 220.10 and 536.10 nm, where PI ranged from 0.21 to 0.58. As expected, the ZP did not vary significantly, since all used materials were of non-ionic nature. Formulation 1 presented the lowest values for Z-Ave and PI, but it was shown to be highly viscous, which would offer difficulties to pass through HPH during the preparation of SLN dispersions, being therefore rejected for further studies. Formulations 3 and 4 (the first formulation developed, bearing 10 wt% solid lipid and 2.5 wt% surfactant) exhibited acceptable particle size values. Thus, as the dependent variables were not statistically different among these two formulations, SLN4 was selected as the optimized formulation due to its lower amount of surfactant.

**[Please insert Table 2 about here]**

**Table 2.** Composition and response values (Z-Ave, PI and ZP) of the three factors depicted in Table 1 for the 7 formulations in study. NiM concentration was the same for all the tested formulations with drug (5 µg/mL). The results are shown as the average value ± standard deviation; n=3.

The analysis of the Pareto charts (Figure S1, supplementary data) depicts the significant effect (p-value < 0.05) of the concentration of solid lipid and surfactant on the Z-Ave (A), and also the significant effect of the concentration of solid lipid in the PI (B). No significant alterations were detected for the ZP of SLN while changing the two independent variables of the factorial design. The limiting factors for the Z-Ave of the SLN were therefore the concentration of solid lipid and the concentration of surfactant, while the former was the variable exhibiting effect on the PI. NiM was further encapsulated into blank SLN in a concentration of 5 µg/mL (NiM-SLN). The loading of particles was successfully achieved, as shown by the maintenance of the nanometric size, low PI and negative value of ZP of NiM-SLN. The particle size distribution of the NiM-loaded formulation was very narrow, being the PI close to 0.1, which represents a monodispersed population. A higher negative value of ZP was obtained for NiM-SLN when compared to the

blank SLN (Table 3), justified by the location of the drug in the inner lipid matrix which shifted the negative charge of the lipids towards the surface of nanoparticles. The physicochemical parameters (Z-Ave, PI and ZP) of SLN and NiM-SLN were evaluated on the day of production and after 7 and 15 days of storage at 4 and 25 °C (Table 3). SLN was shown to be more stable at 4 °C, substantiated by the lower values of the Z-Ave, PI and ZP over the time of the study. The same behaviour was observed for NiM-SLN at the same temperature.

**[Please insert Table 3 about here]**

**Table 3.** Physicochemical parameters of optimized freshly prepared SLN (SLN4) and NiM-SLN dispersions and after 7 and 15 days from this time point at 25 °C and 4 °C (mean ± standard deviation; n = 3).

TurbiscanLab® is used to study reversible (creaming and sedimentation due to fluctuation on particle size and volume) and irreversible destabilization phenomena in the sample (coalescence and segregation facing particle size variation), without the need of further dilution. This approach allows tacking destabilization phenomena based on the measurement of BS and T signals. The evaluation of the physical stability of nanodispersions can be achieved using the TurbiscanLab®. This technique detects particle migration, sedimentation and reversible phenomena. The modification of a BS signal occurs as a function of time and particle migration and is graphically recorded in the form of positive (BS increase) or negative peaks (BS decrease). The migration of particles from the bottom to the top of the sample leads to a progressive concentration decrease at the bottom of the sample and, as a consequence, to a decrease in the BS signal (negative peak) with an increase in the intensity of the T (positive peak). If the BS profiles have a deviation of ≤ ±2%, no significant variations can be considered for Z-Ave. Deviations up to ±10% constitute an indicator of stable formulations [10], whereas above 10% are an indication of unstable formulations. The results depicted in Figure S2 (supplementary data) indicate a higher stability of SLN and NiM-SLN over time, with variations less than 10%.

The cumulative *in vitro* release of NiM was assayed both as free drug solution and as NiM-SLNs (Figure 1). The maximum of drug released after 24 h was ca. 30%, indicating NiM retention into

the NiM-SLN, against 100% of drug quantified in the dialysate within one hour when using the free drug solution. Indeed, for free drug solution the dialysis bag is the rate limiting factor of the release; within 30 minutes, about 50% of drug was found in the receiving compartment, whereas only 2.5% of NiM was released from SLN. Reports on the capacity of SLN in modifying the release profile of loaded drugs are available in literature [15-18]. This property is attributed to the use of lipid materials which recrystallize when cooled down below their melting point. The produced solid matrix offers controlled drug release. Despite the large surface area of the nanoparticles, no burst release was observed.

**[Please insert Figure 1 about here]**

**Figure 1.** Cumulative *in vitro* NiM release profile from NiM-Solution (A) in phosphate buffer (pH 6.8) and from NiM-SLN (B). Values are presented as the mean ± S.D. (n=3).

Freeze-drying is one of the possible methods to increase the shelf-life of labile drugs loaded in SLN [19]. The aim of lyophylization is to obtain a free-flowing powder or a soft ‘‘cake’’ with appropriate characteristics of re-dispersion. Storing of solid products has additional advantages, e.g., easier manipulation, increased water solubility and improvement of the product shelf-life. Freeze-drying of the SLNs dispersions has been shown to be essential for increasing their stability. For this process, a cryoprotectant is needed, which may be a sugar (e.g. trehalose, glucose, mannitol, sorbitol, lactose, sucrose) or a polymer (e.g. polyethylene glycol). The type and amount of cryoprotectant affect the results of the freeze-drying process of SLN dispersions [19]. In this work, three selected cryoprotectants, namely trehalose, sorbitol and mannitol, were tested in different concentrations and proportions aiming the appropriate freeze-drying of NiM-SLN. Freeze-drying of formulations strongly influenced the mean particle size of resuspended SLN powders. Results are nevertheless dependent on the type, concentration and proportion of cryoprotectant:SLN. Absence of cryoprotectant has critically compromised the mean size achieving Z-Ave values of about 7 µm (PI 0.7). Best results were obtained with Trehalose 5% (1:1) 463.1 ± 36.63 nm, followed by Sorbitol 10% (1:1) 743.3 ± 177.1 nm, 5% (1:1) 793.3 ± 38.25 nm, and Manitol 10% (1:1) 815.3 ± 28.78 nm. Trehalose 5% at 1:1 proportion was therefore shown to be the most appropriate cryoprotectant for our SLN, being able of maintaining the lowest values of Z-Ave and PI (Table 4).

**[Please insert Table 4 about here]**

**Table 4.** Particle size, PI and zeta potential for the freeze-dried NiM-SLN. The results are presented as average value ± standard deviation; n=3.

The thermodynamic stability of SLN depends mainly on the lipid modification that occurs after crystallization and on the type of surfactant used [11]. Thus, the physical state and the polymorphic behavior of the developed SLN were studied herein by DSC. The selected solid lipid of the present work is glyceryl behenate, which consists in a mixture of mono-, di- and tribehenate of glycerol [20]. This mixture exhibits a typical crystalline structure, composed of very small α-form, characteristic of triacylglycerols [21]. Triacylglycerols usually occur in three major polymorphic forms, namely α, β' and β, being the α-form the less stable and β the most stable form [22]. In this work, a comparison between the solid lipid, NiM and the obtained SLN formulations (blank SLN, NiM-SLN and freeze-dried NiM-SLN) was conducted. The thermal analysis of the bulk lipid showed a single endothermic peak with a melting point of 82.41 ºC (Table 5). When formulated as lipid nanoparticles, the temperature of the melting event decreased, suffering depression when SLN were loaded with NiM. Re-suspended freeze-dried SLN exhibited a decreased of ca. 19 ºC in the melting peak temperature in comparison to the bulk. These results confirm the polymorphic changes of the solid lipid from the β' form (in the bulk) to the α form in the lipid nanoparticles. With respect to the recrystallinity index (R.I.), which is a measure of the amount of solid content inside SLN, it is perfectly visible the higher solid character of SLN formulations. R.I. (%) is expected to increase with storage time.

**[Please insert Table 5 about here]**

**Table 5:** DSC parameters and recrystalinity index of bulk lipid (glyceryl behenate), blank SLN NiM-SLN, and re-suspended freeze-dried NiM-SLN.

While the effect of the surfactant on the recrystallization of SLN has not been investigated, a crystallization enhancing effect has been described in the case of dispersed lipid material for stabilizers as that of poloxamer used in our samples. Indeed, the surfactant chains act as the ordering principle leading to nucleation at higher temperatures, is often discussed as the cause

for this phenomenon. Even though most of the stabilizers used here have a hydrophobic part that is structurally related to the fatty acid chains of the dispersed triacylglycerols, they do not act as crystallization enhancing templates at the interface. In the case of oil-in-water emulsions containing hydrophobic emulsifier additives with long, saturated fatty acid chains in the oil droplets, the increased crystallization tendency of the dispersed phase has been correlated with the formation of crystalline hydrophobic emulsifier templates in the oil droplets. Previous reports on SLN also describe that rigid surfactant chains are needed to induce crystallization of solid lipid at higher temperatures [23]. The hydrophobic chains of the poloxamer are probably too mobile to induce crystallization at elevated temperatures. The melting behaviour of triacylglycerols nanoparticles strongly depends on particle size, irrespective of matrix material and stabilizer composition. From thermodynamics, it is expected that the melting temperature of colloidal substances would decrease with the decrease of the particle size [24]. Models based on the Thompson equation modified for crystalline materials, or the well-known Gibbs-Thompson equation, are often used to describe the particle size dependence of the melting temperature. They are described using the following equation:

$$-\frac{T_0 - T}{T_0} \approx \ln \frac{T}{T_0} = -\frac{2\,\gamma_{sl}\,V_s}{r\,\Delta H_{fus}}$$

where T is the melting temperature of a particle with radius r, $T_0$ is the melting temperature of the bulk material at the same external pressure, $\gamma_{sl}$ is the interfacial tension at the solid-liquid interface, $V_s$ is the specific volume of the solid, and $\Delta H_{fus}$ is the specific heat of fusion. It has been shown that this equation does not apply for spherical (curved) particles but can also be used to describe the behaviour of particles with plane surfaces. Therefore, the Gibbs-Thompson equation can be used to explain the melting point depression of triacylglycerols nanoparticles compared to their bulk phase. For the size dependence of the melting point of inorganic particles, in particular, modified models are frequently used to account for deviations from the predicted linear relationships between relative melting point depression and particle curvature (1/r) frequently observed with such particles [25]. A shift of the melting transition to lower temperatures with decreasing mean particle size was also observed for the glyceryl behenate dispersions, meaning that the smaller particles melt at lower temperatures. The course of the melting process of small-sized SLN is very unusual once all finely dispersed matrix materials exhibited a more or less stepwise melting event. The structure of very fine particles thus differs significantly from that in the bulk or in coarser colloidal particles. The existence of only one additional, low-melting structure in very fine particles is, however, not sufficient to explain the

complex melting behaviour of the dispersions at higher temperatures. Because of the length of the triacylglycerol chains, the differences in possible platelet heights are of colloidal size, and at least for thin particles, cannot be neglected in relation to the overall platelet height. By assuming that the relation $\gamma_{sl}/r$ in the Gibbs-Thompson equation for crystals is more precisely expressed by $\gamma_x/r_x$ where the relative dimensions of a crystal in equilibrium are interrelated via the interfacial tensions $\gamma_x$ of the corresponding crystal faces by:

$$\frac{\gamma_x}{r_x} = \frac{\gamma_1}{r_1} = \frac{\gamma_2}{r_2} = \frac{\gamma_3}{r_3} = \text{cons tan t}$$

Half of the height of a nanocrystal may be used as the size parameter in the Gibbs-Thompson equation (where $\gamma_x$ is the interfacial tension of the surface x and $r_x$ is the distance between the crystal surface x and the center of the crystal). Since the height of the triacylglycerol nanoparticles can only change in steps corresponding to the thickness of the single triacylglycerol layers reflected by the $d_{001}$ value, this will result in distinct melting temperatures reflecting the differences in particle height. Each of the individual transitions observed in the melting event would thus be due to the melting of a class of particles with a well-defined platelet height.

To confirm the safety of the developed SLN formulations for oral use, cell viability studies have been carried out in human colon adenocarcinoma cell line (Figure 2). The obtained results clearly demonstrate the non-toxic, safety profile of both empty and loaded SLN, with cell viability above 98% after 48h of exposure.

**[Please insert Figure 2 about here]**

**Figure 2:** Caco-2 cell viability after 24 h and 48 h of exposure to different concentrations of nimesulide-free SLN (left) and nimesulide-loaded SLN (right). Results are expressed as a percentage of AlamarBlue® reduction by control cells (non-exposed cells). Data are expressed as arithmetical means ± standard deviations.

## 4. Conclusion

The loading of NSAIDs, as nimesulide (NiM), in SLN has been increasingly reported to circumvent the problem low water solubility of these drug molecules during pharmaceutical development. We have applied a full factorial design to develop an optimized SLN formulation for NiM. This study analyzed the influence of the specific independent variables (concentration of solid lipid and surfactant) on the dependent variables (Z-Ave, PI and ZP). The obtained results revealed the influence of the solid lipid concentration on the Z-Ave and PI; and the influence of the concentration of surfactant on the Z-Ave. The selected blank SLNs formulation was found to be composed by 10 wt% of solid lipid and 2.5 wt% surfactant, and it was characterized for a particle size of 187.8 ± 0.012 nm, PI of 0.199 ± 0.0120 and ZP of -2.10 ± 0.247 mV. The encapsulation of NiM into SLNs accounted for a new formulation with a particle size of 166.1 ± 0.114 nm, PI of 0.171 ± 0.051 and ZP of -3.10 ± 0.166 mV, highlighting the successful encapsulation of the drug. Stability studies disclosed the higher stability of both SLN and NiM-SLN when stored at 4 °C (refrigerated conditions). The release of NiM from NiM-SLN was shown to be slow, releasing up to 30% of the loaded drug after 24 h. The best results arising from the addition of a cryoprotectant for the freeze-drying process were obtained when using trehalose 5 wt% at 1:1 proportion. DSC demonstrated the solid character of SLN both after encapsulation of NiM and after resuspended freeze-dried SLN. The long-term stability studies showed that SLN and NiM-SLN were physically stable, attributed to changes below 10% in TurbiscanLab®. These results make proof of the present optimized formulation as a promising oral formulation for NiM.

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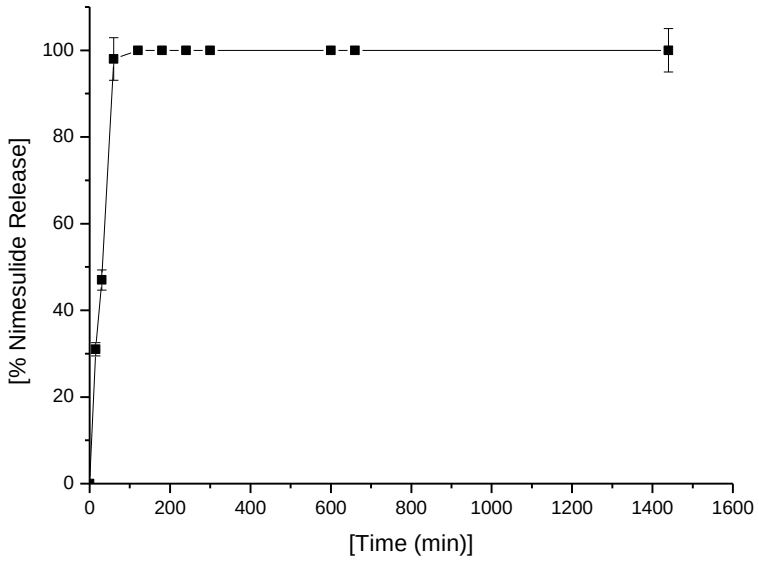
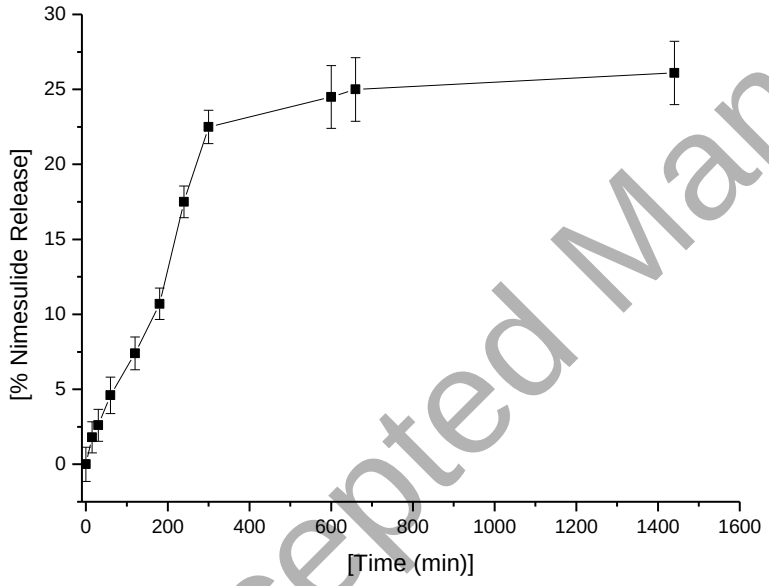
**Figure Captions**

**Figure 1.** Cumulative *in vitro* NiM release profile from NiM-Solution (A) in phosphate buffer (pH 6.8) and from NiM-SLN (B). Values are presented as the mean ± S.D. (n=3).

**Figure 2:** Caco-2 cell viability after 24 h and 48 h of exposure to different concentrations of nimesulide-free SLN (left) and nimesulide-loaded SLN (right). Results are expressed as a percentage of AlamarBlue® reduction by control cells (non-exposed cells). Data are expressed as arithmetical means ± standard deviations.




(A)




(B)



**Figure 1.** Cumulative *in vitro* NiM release profile from NiM-Solution (A) in phosphate buffer (pH 6.8) and from NiM-SLN (B). Values are presented as the mean ± S.D. (n=3).

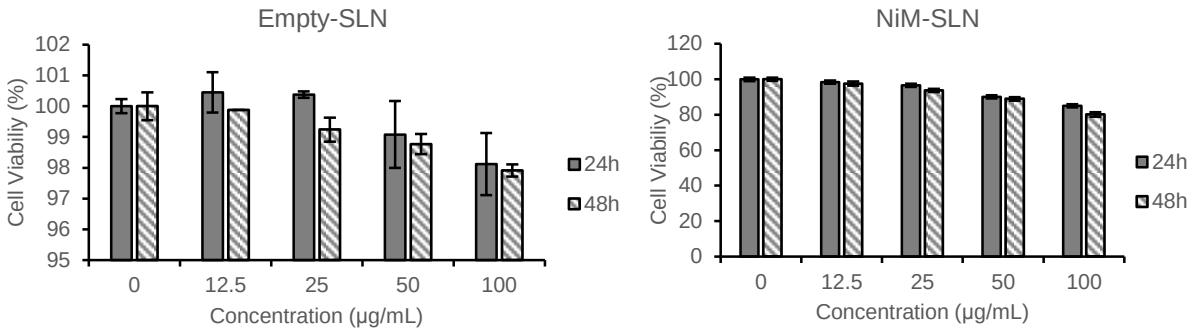



**Figure 2:** Caco-2 cell viability after 24 h and 48 h of exposure to different concentrations of nimesulide-free SLN (left) and nimesulide-loaded SLN (right). Results are expressed as a percentage of AlamarBlue® reduction by control cells (non-exposed cells). Data are expressed as arithmetical means ± standard deviations.

**Table Captions**

**Table 1.** Independent variables and levels used in the factorial design.

**Table 2.** Composition and response values (Z-Ave, PdI and ZP) of the three factors present in Table 1 for the 7 formulations in study. NiM concentration was the same for all the tested formulations with drug (5 mg/mL).The results are presented as average value ± standard deviation; n=3.

**Table 3.** Physicochemical parameters of optimized freshly prepared SLN (SLN4) and NiM-SLN dispersions and after 7 and 15 days from this time point at 25 °C and 4 °C (mean ± standard deviation; n = 3).

**Table 4.** Particle size, PI and zeta potential for the freeze-dried NiM-SLN. The results are presented as average value ± standard deviation; n=3.

**Table 5:** DSC parameters and recrystalinity index of bulk lipid (glyceryl behenate), blank SLN NiM-SLN, and re-suspended freeze-dried NiM-SLN.

**Table 1.** Independent variables and levels used in the factorial design.

| | ***Low level*** | ***Medium level*** | ***High level*** |
|---|---|---|---|
| **Independent Variables** | -1 | 0 | 1 |
| [Glyceryl behenate] (wt%) | 20 | 15 | 10 |
| [Poloxamer 188] (wt%) | 3.5 | 3.0 | 2.5 |

**Table 2.** Composition and response values (Z-Ave, PdI and ZP) of the three factors depicted in Table 1 for the 7 formulations in study. NiM concentration was the same for all the tested formulations with drug (5 mg/mL). The results are shown as the average value ± standard deviation; n=3.

| **Assayed formulations** | **Independent variables** | | **Dependent variables** | | |
|---|---|---|---|---|---|
| | **[Glyceryl behenate] (wt%)** | **[Poloxamer 188] (wt%)** | **Z-Ave (nm) (nm)** | **PdI** | **ZP (mV)** |
| SLN1 | 20 | 3.5 | 220.10±8.36 | 0.21± 0.013 | -1.40± 0.003 |
| SLN2 | 10 | 3.5 | 536.10± 12.11 | 0.58± 0.011 | -1.60± 0.000 |
| SLN3 | 20 | 2.5 | 271.70± 6.34 | 0.25± 0.043 | -1.00± 0.009 |
| SLN4 (Optimal) | 10 | 2.5 | 275.30± 6.01 | 0.39± 0.012 | -1.60± 0.004 |
| SLN5 (Central point) | 15 | 3.0 | 381.00± 9.03 | 0.49± 0.059 | -1.59± 0.001 |
| SLN6 (Central point) | 15 | 3.0 | 327.50± 10.11 | 0.42± 0.050 | -1.64± 0.005 |
| SLN7 (Central point) | 15 | 3.0 | 332.50± 7.54 | 0.35± 0.011 | -1.68± 0.000 |

**Table 3.** Physicochemical parameters of optimized freshly prepared SLN (SLN4) and NiM-SLN dispersions and after 7 and 15 days from this time point at 25 °C and 4 °C (mean ± standard deviation; n = 3).

| **Formulation** | **Day** | **Storage temperature** | **Z-Ave (nm)** | **PdI** | **ZP (mV)** |
|---|---|---|---|---|---|
| SLN | d0 | Freshly prepared | 187.8 ± 0.012 | 0.199 ± 0.0120 | -2.10 ± 0.247 |
| | d7 | 25 °C | 272.7 ± 9.370 | 0.455 ± 0.027 | -2.20 ± 0.113 |
| | | 4 °C | 199.8 ± 5.700 | 0.238 ± 0.012 | -2.21 ± 0.198 |
| | d15 | 25 °C | 218.1 ± 2.458 | 0.316 ± 0.035 | -2.39 ± 0.134 |
| | | 4 °C | 196.0 ± 3.051 | 0.254 ± 0.011 | -2.35 ± 0.146 |
| NiM-SLN | d0 | Freshly prepared | 166.1 ± 0.114 | 0.171 ± 0.051 | -3.10 ± 0.166 |
| | d7 | 25 °C | 202.8 ± 0.436 | 0.455 ± 0.033 | -2.43 ± 0.007 |
| | | 4 °C | 173.1 ± 1.700 | 0.238 ± 0.024 | -2.99 ± 0.139 |
| | d15 | 25 °C | 211.0 ± 0.458 | 0.316 ± 0.029 | -2.83 ± 0.064 |
| | | 4 °C | 184.2 ± 1.143 | 0.254 ± 0.031 | -2.55 ± 0.155 |

**Table 4.** Particle size, PI and zeta potential for the freeze-dried NiM-SLN. The results are presented as average value ± standard deviation; n=3.

| Cryoprotectant | Proportion (Cryoprotectant:SLN) | Z-Ave (nm) | PdI | ZP (mV) |
|---|---|---|---|---|
| Trehalose 5% | 1:1 | 463.1 ± 36.63 | 0.491 ± 0.0270 | -2.26 ± 0.0778 |
| | 2:1 | 1273 ± 403.7 | 0.868 ± 0.187 | -2.15 ± 0.382 |
| Trehalose 10% | 1:1 | 2259 ± 653.4 | 1.000 ± 0.000 | -2.95 ± 0.156 |
| | 2:1 | n.d. | n.d. | n.d. |
| Sorbitol 5% | 1:1 | 793.3 ± 38.25 | 0.803 ± 0.0350 | -1.57 ± 0.410 |
| | 2:1 | n.d. | n.d. | n.d. |
| Sorbitol 10% | 1:1 | 743.3 ± 177.1 | 0.660 ± 0.0280 | -2.44 ± 0.256 |
| | 2:1 | 1550 ± 776.3 | 0.653 ± 0.337 | -2.73 ± 0.205 |
| Mannitol 5% | 1:1 | 1745 ± 646.3 | 1.000 ± 0.000 | -3.57 ± 0.502 |
| | 2:1 | 1525 ± 632.2 | 1.000 ± 0.000 | -2.86 ± 0.710 |
| Mannitol 10% | 1:1 | 815.3 ± 28.78 | 0.811 ± 0.0590 | -1.94 ± 0.354 |
| | 2:1 | 1700 ± 804.7 | 0.645 ± 0.302 | -2.44 ± 0.289 |
| Without cryoprotectant | | 6923 ± 3620 | 0.720 ± 0.283 | -0.820 ± 0.0367 |

n.d., not determined (semi-solid system).

**Table 5:** DSC parameters and recrystallinity index of bulk lipid (glyceryl behenate), blank SLN NiM-SLN, and re-suspended freeze-dried NiM-SLN.

| **Samples** | **Melting point (ºC)** | **Onset (ºC)** | **R.I. (%)** |
|---|---|---|---|
| Glyceryl behenate | 82.41 | 80.29 | 100 |
| Blank SLN (Nr.4) | 74.12 | 73.77 | 89.45 |
| NiM-SLN | 73.23 | 72.83 | 83.11 |
| NiM-SLN (freeze-dried) | 63.85 | 61.65 | 74.24 |