



## Solid lipid nanoparticles optimized by 2<sup>2</sup> factorial design for skin administration: Cytotoxicity in NIH3T3 fibroblasts

Roberta Balansin Rigon<sup>a,1</sup>, Maíra Lima González<sup>a,1</sup>, Patrícia Severino<sup>b</sup>, Danilo Antonini Alves<sup>c</sup>, Maria H.A. Santana<sup>d</sup>, Eliana B. Souto<sup>e,f,\*</sup>, Marlus Chorilli<sup>a,\*\*</sup>

<sup>a</sup> Faculty of Pharmaceutical Sciences, UNESP – São Paulo State University, Campus Araraquara, Departamento de Fármacos e Medicamentos, Araraquara, SP 14800-850, Brazil

<sup>b</sup> Centre of Biological Sciences and Health, Tiradentes University, Aracaju, Sergipe 49010-390, Brazil

<sup>c</sup> Laboratory of Biotechnology, Institute of Biology, Campinas University (UNICAMP), Campinas, São Paulo 13083-862, Brazil

<sup>d</sup> Faculty of Chemical Engineering, Campinas University (UNICAMP), Campinas, São Paulo 13083-970, Brazil

<sup>e</sup> Department of Pharmaceutical Technology, Faculty of Pharmacy, University of Coimbra (FFUC), Polo das Ciências da Saúde Azinhaga de Santa Comba, 3000-548 Coimbra, Portugal

<sup>f</sup> REQUIMTE/LAQV, Group of Pharmaceutical Technology, Faculty of Pharmacy, University of Coimbra, Portugal

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### ABSTRACT

The present study focuses on the characterization of the cytotoxic profile on NIH3T3 **mouse embryonic** fibroblasts of solid lipid nanoparticles (SLN) optimized by a 2<sup>2</sup> full factorial design for skin administration. To build up the surface response charts, a design of experiments (DoE) based on 2 independent variables was used to obtain an optimized formulation. The effect of the composition of lipid and water phases on the mean particle size (z-AVE), polydispersity index (PdI) and zeta potential (ZP) was **studied**. The **developed formulations** were composed of 5.0% of lipid phase (stearic acid (SA), behenic alcohol (BA) or a blend of SA:BA (1:1)) and 4.7% of surfactants (soybean phosphatidylcholine and poloxamer 407). In vitro cytotoxicity using **NIH3T3** fibroblasts was performed by MTT reduction assay. This factorial design study has proven to be a useful tool in optimizing SLN (z-AVE ~ 200 nm), which were shown to be non-cytotoxic. The present results highlight the benefit of applying statistical designs in the preparation and **optimization** of SLN **formulations**.

### 1. Introduction

Solid lipid nanoparticles (SLN) have been widely used for skin delivery of active pharmaceutical ingredients (APIs) due to their safe interaction with stratum corneum and other skin layers, and improved skin permeation [1,2]. Additional attributes include the possibility of controlled release of the loaded APIs, drug protection, biodegradability and safety profile, and low cost of the production process [3–5]. For this purpose, a large number of lipid ingredients may be used, including glycerol behenate, glycerol palmitostearate, cetyl palmitate, glycerol trilaurate, and stearic acid as solid lipids, which are known to have approved safety profile for a set of clinical applications [3,4]. For the physicochemical stability of SLN, several surfactants may be employed e.g. sodium chlorate, polysorbates, phospholipids and poloxamers,

polyvinyl alcohols, which should also be of generally recognized as safe status.

The development of a new formulation encounters numerous variables which have to be taken into account when loading a new API. Factorial design experiments offer a simple, efficient and statistically valid approach which can simultaneously analyze the influence of different variables on the properties of drug delivery systems [18]. In this way, optimization with respect to the selection of ingredients and methodology parameters to prepare the formulation can be achieved by factorial design, with the ultimate aim of reducing the time for the development of a new formulation, as well as the production costs [19].

The aim of this study **has been the optimization** by factorial design of an innovative SLN formulation composed of stearic acid (SA) and/or behenic alcohol (BA) as solid lipids and stabilized by a combination of

\* Corresponding author at: Department of Pharmaceutical Technology, Faculty of Pharmacy, University of Coimbra (FFUC), Pólo das Ciências da Saúde, Azinhaga de Santa Comba, 3000-548 Coimbra, Portugal.

\*\* Corresponding author at: Faculty of Pharmaceutical Sciences, UNESP – São Paulo State University, Campus Araraquara, Departamento de Fármacos e Medicamentos, Araraquara, SP 14800-850, Brazil.

E-mail addresses: [souto.eliana@gmail.com](mailto:souto.eliana@gmail.com), [ebsouto@ff.uc.pt](mailto:ebsouto@ff.uc.pt) (E.B. Souto), [chorilli@fcar.unesp.br](mailto:chorilli@fcar.unesp.br) (M. Chorilli).

<sup>1</sup> These authors contributed equally to this work.

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poloxamer 407 (P407) and soy phosphatidylcholine (SP), for skin application. The dependent variables were set as the mean hydrodynamic diameter, polydispersity index (PDI) and zeta potential (ZP), while the ratio of lipids and surfactants were defined as the independent variables.

## 2. Materials and methods

### 2.1. Materials

Stearic acid (SA) was obtained from Via Farma (São Paulo, Brazil), behenic alcohol (BA) and poloxamer 407 (Pluronic® F127) were purchased from Sigma-Aldrich (St. Louis, USA), and soybean phosphatidylcholine (Lipoid® S100) from Lipoid GmbH (Ludwigshafen, Germany). NIH 373 (ATCC® CRL-1658™) were obtained from LGC Standards S.L.U. (Barcelona, Spain). RPMI 1640 was purchased from Lonza (Verviers, Belgium), and Fetal Bovine Serum from Biowest (Nuaillé, France). Double distilled water was used after filtration in a Millipore system (home supplied).

### 2.2. Methods

#### 2.2.1. Preparation of solid lipid nanoparticles

SLN composed of 5.0% of lipid phase (SA or BA, or a blend of SA and BA) and 4.7% of surfactants (SP and P407) were produced by high shear homogenization. Briefly, the lipid phase (solid lipid + SP) was heated up to approximately 5–10 °C above its melting point, before being added to the aqueous surfactant solution of poloxamer P407 of the same temperature. The formulations were stirred for 1 min using a magnetic stirrer. Then, the SLN mixture was sonicated using an ultrasonic processor (Q700 Sonicator, QSonica Sonicators, USA) for 20 min (amplitude 6 µm, 14–19 W power, 20 kHz frequency, ½" probe). During sonication, samples were maintained in a cooling bath. **Since the sonication process encounters the risk of titanium contamination, samples were centrifuged at 5000 rpm for 10 min to remove any metal traces [6]. As SLN are covered with surfactant, they remained in suspension with the pre-set centrifugation conditions.**

Factorial design was used to optimize the production yield, requiring a minimum of experiments. The influence of the lipid nanoparticles' composition on their mean hydrodynamic diameter (z-AVE), polydispersity index (PDI) and zeta potential (ZP) was evaluated using a 2<sup>2</sup> factorial design, i.e. 2 variables which were set at 2-levels each, with central point for estimating the experimental error requiring a total of 5 experiments. The lower and higher values of the lower and upper levels are represented by (– 1) and (+ 1) and the central point by (0), as summarized in Table 1.

#### 2.2.2. Particle size parameters and zeta potential

The mean hydrodynamic diameter (z-AVE), polydispersity index (PDI) and zeta potential (ZP) of SLN were determined by photon correlation spectroscopy i.e. Dynamic Light Scattering (DLS, Zetasizer Nano NS, Malvern Instruments, Malvern, UK). The samples were diluted in ultra-purified water (10 µL/mL) to attenuate their opalescence

**Table 1**

The 2<sup>2</sup> factorial design used for the development of an optimized SLN formulation, providing the lower (– 1), upper (+ 1) and (0) central point level.

Variable	Level		
	– 1	0	+ 1
SA:BA <sup>a</sup>	1:0	1:1	0:1
P407:SP <sup>b</sup>	3.7:1.0	3.5:1.2	3.3:1.4

<sup>a</sup> 5.0% of solid lipid, which a mixture of stearic acid: behenic alcohol.

<sup>b</sup> 4.7% of surfactant in aqueous solution, which a mixture of poloxamer 407: soybean phosphatidylcholine.

placed in scintillation vials to maintain cleanliness. All the analyses were done in triplicate (n = 3) and data are given as the average values and standard deviations.

#### 2.2.3. In vitro cytotoxicity assay

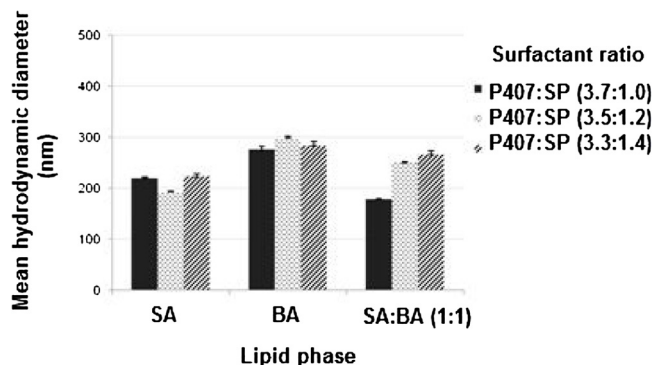
In vitro cytotoxicity assays of SLN composed of 5.0% of SA and 3.1:1.4 P407:SP was performed using NIH 373 mouse fibroblasts as cell model. Cells were seeded in 96-well plates at a density of 10 × 10<sup>3</sup> cells/well with different concentrations of SLN (7.0–125.0 µg/mL) at 37 °C and 5.0% of CO<sub>2</sub> for 24 h, or medium without SLN as vehicle control or medium without nutrient as negative control. The cells were washed with phosphate-buffered saline (PBS) and cell viability was assessed by MTT reduction assay [7,8]. After removal of the treatment media and cells washed with PBS, the MTT solution (0.5 mg/mL of culture medium) was added to each well and cells were seeded at 37 °C for 3 h. MTT solution was then removed and formazan crystals solubilized in 100 µL of ethanol. The plates were shaken for 10 min on a plate shaker and the absorbance was measured at 570 nm in a microplate reader (Spectrophotometer Infinite® 200 PRO series).

## 3. Results and discussion

The effect of the lipid composition (i.e. SA, BA or a blend of SA:BA at ratio 1:1) and surfactant ratio on the mean hydrodynamic diameter of SLN is shown in Fig. 1, with recorded z-AVE values between 180 and 300 nm. At a first glance, the lowest mean particle size was recorded for the SLN produced with a combination of SA:BA at ratio 1:1, stabilized by P407:SP (3.7:1.0). Table 2 shows the influence of the lipid (SA:BA) and surfactant (P407:SP) ratio on the polydispersity index (PDI) and zeta potential (ZP) of SLN.

To determine the mean hydrodynamic diameter responses, the following equation has been used:

Hydrodynamic diameter (nm) = 250.92 + 58.15\*[lipid]<sup>a</sup> + 5.7\*[surfactants]<sup>b</sup> where <sup>a</sup> stands for the ratio between the lipid phases (SA:BA) and <sup>b</sup> for ratio between the surfactants (P407:SP) [9]. Indeed, a frequent problem observed in pre-formulation studies is optimizing the excipients concentration in order to obtain a final formulation with the required attributes [10]. From the equation, the mean particle size was 58.15-fold more influenced by the ratio between the two selected lipids (SA:BA), whereas the ratio between both surfactants influenced only 5.7 times. These results are corroborated by those shown in Fig. 2a, which demonstrate that the ratio between the percentage of surfactants (P407:SP) exhibited low influence on the mean hydrodynamic diameter of the obtained SLN, but nevertheless also statistically significant (Fig. 3a). With respect to the ratio between both lipids (SA:BA), the increase on the mean hydrodynamic diameter of nanoparticles was shown to be directly proportional to BA concentration, i.e. a higher mean hydrodynamic diameter was obtained when SLN were composed



**Fig. 1.** Effect of the lipid composition (i.e. SA or BA or a blend of SA:BA at ratio 1:1) and surfactant ratio on mean hydrodynamic diameter of SLN. Error bars stand for standard deviation (SD, n = 3).

**Table 2**

Influence of the lipid (SA:BA) and surfactant (P407:SP) ratio on the polydispersity index (PDI) and zeta potential (ZP) of SLN.

SA:BA	P407:SP	PdI	SD	ZP (mV)	SD
–1	–1	0.173	0.010	–22.2	0.289
+1	–1	0.187	0.022	–19.2	0.115
0	0	0.276	0.018	–33.1	0.252
–1	+1	0.194	0.021	–23.5	0.200
+1	+1	0.174	0.018	–13.6	0.173

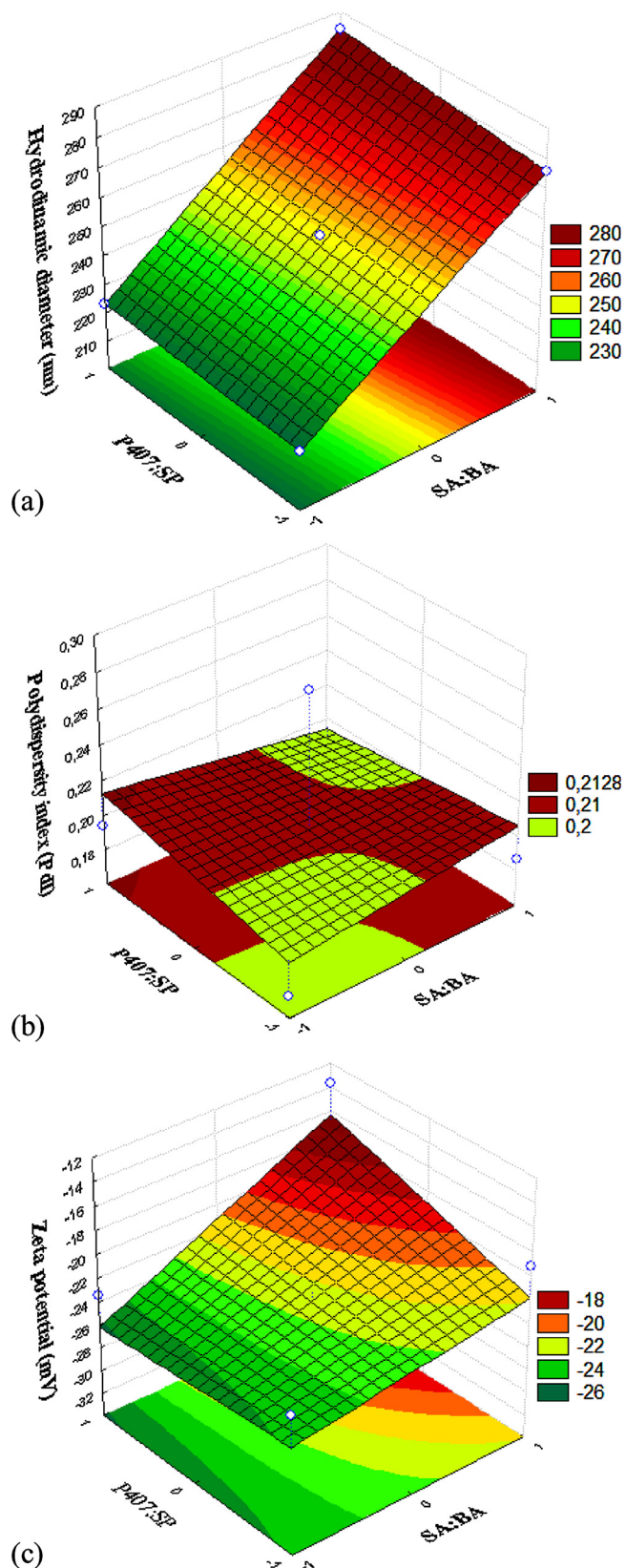
SD: standard deviation.

only of BA as solid lipid (Equation 1). These results were attributed to the different viscosities of lipids used, i.e. SA ( $C_{18}H_{36}O_2$ ) has 7.79 mPa/s and BA ( $C_{22}H_{46}O$ ) has 40.4 mPa/s. BA can increase the formulation viscosity and when it is combined with polymers the viscosity is even higher [11]. Viscous formulations usually show higher polydispersity and larger mean particle size, attributed to the lower capacity for energy distribution during the homogenization process [12,13]. On the other hand, it has also been reported that the increased viscosity in nano-formulations might not have a direct influence on the increase of the particle size, but rather the enhanced risk of formation of agglomerates and/or aggregates [14]. With respect to the production of SLN, the particle size distribution is highly dependent on the lipid and surfactant composition, viscosity of the aqueous phase and production parameters [15]. Jennings et al. conducted a study that aimed to verify the influence of the viscosity of lipid on the particle size [16]. Leaving all other parameters constant, and varying only the composition of the lipid matrix, the results demonstrate that lipid phase of low viscosity improved the homogenization process. In addition, the processing of lipids of low viscosity at room temperature could ameliorate the particle size distribution when using an incompletely thermos-regulated homogenizer with colder spots, an effect that has not been observed when homogenizing the lipid phase at 85 °C. Hu et al. demonstrated that the addition of a liquid lipid (e.g. oleic acid) to stearic acid SLN could decrease the viscosity of the lipid phase thereby reducing the PDI in a proportional fashion with respect to the oleic acid content [17].

None of our tested variables had a significant effect on the PDI of the SLN (Figs. 2b and 3b). For all SLN matrices, the range of PDI was between 0.190 and 0.217, which can be classified as a monomodal distribution (Figs. 2b and 3b). Indeed, a nanoparticle formulation can display a monomodal (only one population) versus plurimodal (multiple populations), and monodisperse (narrow distribution) versus polydisperse (wide distribution). As PDI is used to describe particle size distribution, its values vary between 0 and 1. Thus, PDI lower than 0.1 is associated to a higher uniformity in particle size distribution, whereas PDI higher than 0.5 suggests a wide particle size distribution or the presence of multiple populations [15,18].

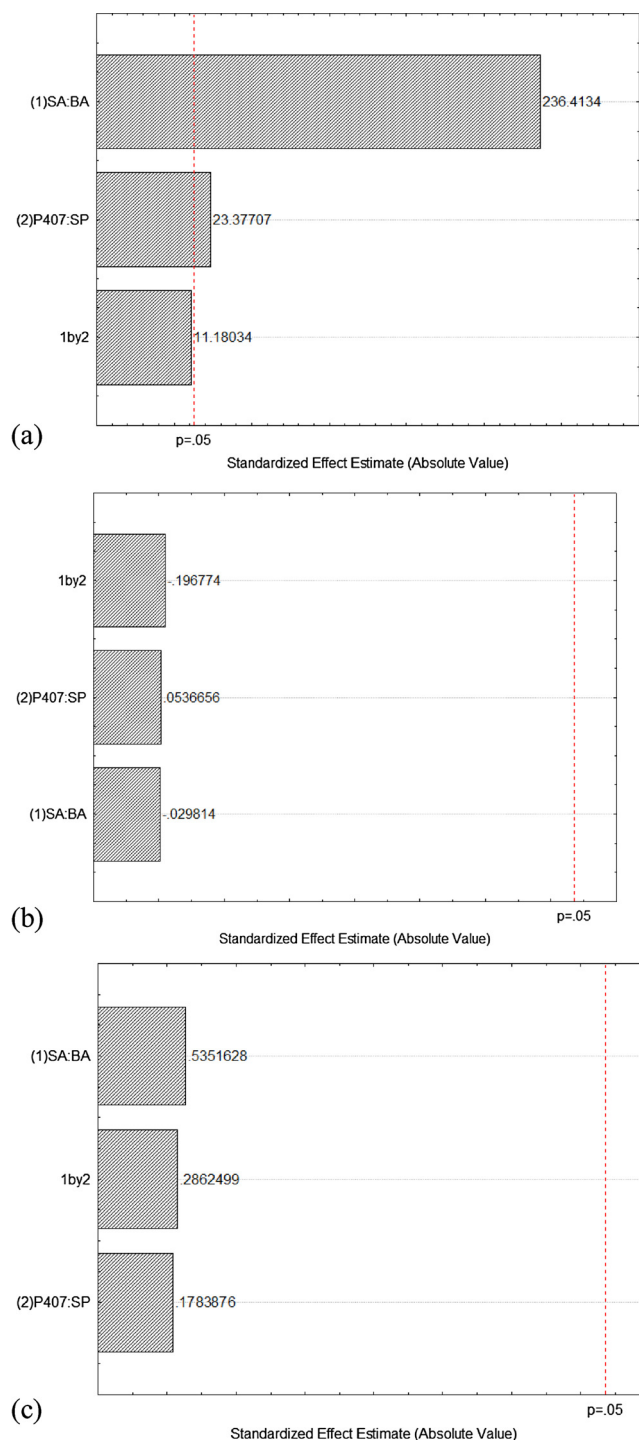
Decreasing the P407:SP ratio (i.e. decrease of %P407 and increase of %SP) increased the absolute values of the ZP for formulation composed only of SA (Fig. 2c), but without a significant effect on the mean particle size (Fig. 3c). On the other hand, when increasing the %BA (and decreasing %SA) a decrease of the ZP was observed. Particles with zeta potential higher than +30 mV or lower than –30 mV are considered physically stable [19]. Nevertheless, good electrostatic stabilization could also be achieved with absolute values of zeta potential between 8 and 9 mV [20]. While all formulations presented a minimum value for electrostatic stabilization, that composed only of SA and 3.3:1.4 P407:SP ratio demonstrated better results with respect to the zeta potential values (close to  $\pm 30$  mV).

Cytotoxicity of the selected formulation (5.0% of SA and 4.7% of surfactants P407:SP at a ratio 3.1:1.4) was determined by calculating total number of viable cells in SLN-treated wells compared to the total number of viable cells in untreated control wells (positive control). High percentage of cellular viability indicates low toxicity (10%, high



**Fig. 2.** Surface response charts of experimental design: (a) mean hydrodynamic diameter (nm); (b) polydispersity index; (c) zeta potential as a function of SA:BA (stearic acid: behenic alcohol) and P407:SP (poloxamer 407:soybean phosphatidylcholine) ratio.

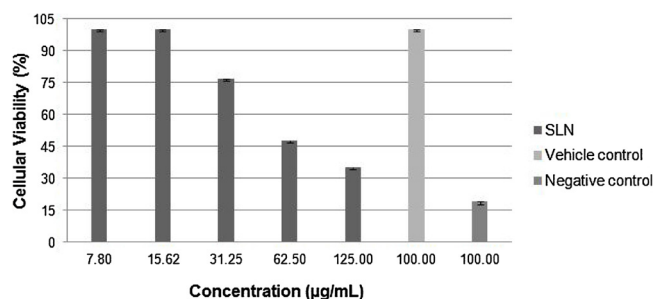




**Fig. 3.** Pareto charts of the standardized effects: (a) hydrodynamic diameter (nm); (b) polydispersity index; (c) zeta potential as a function of SA:BA (stearic acid: behenic alcohol) and P407:SP (poloxamer 407:soybean phosphatidylcholine) ratio.

toxicity; 11%–40%, moderate toxicity; 40%–70%, low toxicity;  $\geq 70\%$  without toxicity) [21]. NIH3T3 mouse embryonic fibroblast cells served as standard cell line for evaluating in vitro cytotoxicity of skin delivery formulations [22]. Results are shown as the percentage of viable cells (Fig. 4).

Cytotoxicity of the tested SLN was shown to follow a concentration-dependent profile, i.e. SLN composed of 5.0% SA and 3.3:1.4 P407:SP was not cytotoxic up to 31.25  $\mu\text{g/mL}$  with  $76.55\% \pm 0.009$  of cellular viability. Duplicating the concentration (62.50  $\mu\text{g/mL}$ ), cell viability



**Fig. 4.** Percentage of cell viability after treatment with SLN (5.0% SA and 3.3:1.4 P407:SP), with vehicle control (medium without SLN) and with negative control (medium without nutrients). Error bars stand for standard deviation (SD,  $n = 3$ ).

was reduced down to ca. 47%, while with the highest tested concentration (125  $\mu\text{g/mL}$ ) the percentage of viable cells was as low as  $34.95\% \pm 0.0171$  (moderate toxicity). The observed reduction of viable cells was attributed to cells hypoxia, as the high concentration of lipid promotes insufficient oxygen supply. However, any concentration demonstrated high toxicity, only moderate toxicity was observed ( $34.95\% \pm 0.0171$  of cellular viability).

#### 4. Conclusions

We have demonstrated that a simple factorial design approach, based on two dependent variables, was useful for the optimization of solid lipid nanoparticles produced by sonication. Nanoparticles with higher percentage of stearic acid and lower P407:SP ratio (3.3:1.4) presented the smallest hydrodynamic diameter, with 0.200 of PDI and ZP values of  $[-26 \text{ mV}]$ . When treating NIH3T3 fibroblast cells, SLN did not demonstrate relevant cytotoxic events at concentrations up to 39  $\mu\text{g/mL}$ , translating the suitability of the developed formulation for skin administration.

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#### References

- [1] D. Guo, D. Dou, X. Li, Q. Zhang, Z.A. Bhutto, L. Wang, Ivermectin-loaded solid lipid nanoparticles: preparation, characterisation, stability and transdermal behaviour, *Artif. Cells Nanomed. Biotechnol.* (2017) 1–8.
- [2] S.N. Shrotriya, N.S. Ranpise, B.V. Vidhate, Skin targeting of resveratrol utilizing solid lipid nanoparticle-engrossed gel for chemically induced irritant contact dermatitis, *Drug Deliv. Transl. Res.* 7 (2017) 37–52.
- [3] S. Doktorovova, A.B. Kovacevic, M.L. Garcia, E.B. Souto, Preclinical safety of solid lipid nanoparticles and nanostructured lipid carriers: current evidence from in vitro and in vivo evaluation, *Eur. J. Pharm. Biopharm.* 108 (2016) 235–252.
- [4] S. Doktorovova, E.B. Souto, A.M. Silva, Nanotoxicology applied to solid lipid nanoparticles and nanostructured lipid carriers - a systematic review of in vitro data, *Eur. J. Pharm. Biopharm.* 87 (2014) 1–18.
- [5] E.B. Souto, S. Doktorovova, Chapter 6 - Solid lipid nanoparticle formulations pharmacokinetic and biopharmaceutical aspects in drug delivery, *Meth. Enzymol.* 464 (2009) 105–129.
- [6] R.R. Agayan, T. Horvath, B.H. McNaughton, J.N. Anker, R. Kopelman, Optical manipulation of metal-silica hybrid nanoparticles, *Optical Science and Technology, the SPIE 49th Annual Meeting: International Society for Optics and Photonics*,

- (2004), pp. 502–513.
- [7] M.L. Gonzalez, M.A. Correa, M. Chorilli, Skin delivery of kojic acid-loaded nanotechnology-based drug delivery systems for the treatment of skin aging, *Biomed Res. Int.* (2013) (2013) 271276.
  - [8] M.B. Oliveira, A.H. do Prado, J. Bernegossi, C.S. Sato, I. Lourenco Brunetti, M.V. Scarpa, G.R. Leonardi, S.E. Friberg, M. Chorilli, Topical application of retinyl palmitate-loaded nanotechnology-based drug delivery systems for the treatment of skin aging, *Biomed Res. Int.* (2014) (2014) 632570.
  - [9] E. Acosta, L. Diosady, A.V. Rao, D. Rousseau, C. Yip, Self-emulsifying delivery systems and lipid transport, in: Moghis U. Ahmad (Ed.), *Lipids in Nanotechnology*, 2012, pp. 135–170 Chapter 6.
  - [10] B. Campisi, D. Chicco, D. Vojnovic, R. Phan-Tan-Luu, Experimental design for a pharmaceutical formulation: optimisation and robustness, *J. Pharm. Biomed. Anal.* 18 (1998) 57–65.
  - [11] K.R. Olesen, D.R. Bassett, C.L. Wilkerson, Surfactant co-thickening in model associative polymers, *Prog. Org. Coat.* 35 (1999) 161–170.
  - [12] N. Innocente, M. Biasutti, E. Venir, M. Spaziani, G. Marchesini, Effect of high-pressure homogenization on droplet size distribution and rheological properties of ice cream mixes, *J. Dairy Sci.* 92 (2009) 1864–1875.
  - [13] W. Mehnert, K. Mader, Solid lipid nanoparticles: production, characterization and applications, *Adv. Drug Deliv. Rev.* 47 (2001) 165–196.
  - [14] M.A. Osman, A. Atallah, Effect of the particle size on the viscoelastic properties of filled polyethylene, *Polymer* 47 (2006) 2357–2368.
  - [15] C. Schwarz, W. Mehnert, Solid lipid nanoparticles (SLN) for controlled drug delivery. II. Drug incorporation and physicochemical characterization, *J. Microencapsul.* 16 (1999) 205–213.
  - [16] V. Jennings, A.F. Thunemann, S.H. Gohla, Characterisation of a novel solid lipid nanoparticle carrier system based on binary mixtures of liquid and solid lipids, *Int. J. Pharm.* 199 (2000) 167–177.
  - [17] F.Q. Hu, S.P. Jiang, Y.Z. Du, H. Yuan, Y.Q. Ye, S. Zeng, Preparation and characterization of stearic acid nanostructured lipid carriers by solvent diffusion method in an aqueous system, *Colloids Surf. B Biointerfaces* 45 (2005) 167–173.
  - [18] M. Gaumet, A. Vargas, R. Gurny, F. Delie, Nanoparticles for drug delivery: the need for precision in reporting particle size parameters, *Eur. J. Pharm. Biopharm.* 69 (2008) 1–9.
  - [19] S. Jose, S.S. Anju, T.A. Cinu, N.A. Aleykutty, S. Thomas, E.B. Souto, In vivo pharmacokinetics and biodistribution of resveratrol-loaded solid lipid nanoparticles for brain delivery, *Int. J. Pharm.* 474 (2014) 6–13.
  - [20] E. Zimmermann, R.H. Muller, Electrolyte- and pH-stabilities of aqueous solid lipid nanoparticle (SLN) dispersions in artificial gastrointestinal media, *Eur. J. Pharm. Biopharm.* 52 (2001) 203–210.
  - [21] H.M. Shubar, J.P. Mayer, W. Hopfenmuller, O. Liesenfeld, A new combined flow-cytometry-based assay reveals excellent activity against *Toxoplasma gondii* and low toxicity of new bisphosphonates in vitro and in vivo, *J. Antimicrob. Chemother.* 61 (2008) 1110–1119.
  - [22] J.M. Hosmer, S.H. Shin, A. Nornoo, H. Zheng, L.B. Lopes, Influence of internal structure and composition of liquid crystalline phases on topical delivery of paclitaxel, *J. Pharm. Sci.* 100 (2011) 1444–1455.