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Cytotoxicity of submicron emulsions and solid lipid nanoparticles for dermal application

Wim Weyenberg [a], Plamen Filev [a], Dave Van den Plas [b], Jo Vandervoort [a], Kris De Smet [b], Philippe Sollie [b], Annick Ludwig [a,*]

[a] *Laboratory of Pharmaceutical Technology and Biopharmacy, University of Antwerp, Universiteitsplein 1, B-2610 Antwerp, Belgium*
[b] *Flen Pharma NV, Drie Eikenstraat 661, B-2650 Antwerp, Belgium*

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Abstract

The cytotoxicity and physical properties of various submicron O/W emulsions and solid lipid nanoparticles for dermal applications were investigated. Droplet size and zetapotential of submicron emulsions depended on the composition of the cosurfactant blend used. The viability of J774 macrophages, mouse 3T3 fibroblasts and HaCaT keratinocytes was significantly reduced in the presence of stearylamine. Nanoparticles consisting of stearic acid or different kinds of adeps solidus could be manufactured when formulated with lecithin, sodium taurocholate, polysorbate 80 and stearylamine. Survival of macrophages was highly affected by stearic acid and stearylamine. In general a viability of more than 90% was observed when semi-synthetic glycerides or hard fat was employed to formulate nanoparticles.

Keywords: Cytotoxicity; Solid lipid nanoparticles; Submicron emulsion; Particle size; Zetapotential

1. Introduction

Submicron emulsions and solid lipid nanoparticles (SLN) have gained widespread interest in recent years as drug carriers for dermatological purpose, aiming a better permeation or prolonged action on the skin or in specific skin layers (Müller et al., 2002; Cevc, 2004; Lombardi Borgia et al., 2005; Üner, 2006; Chen et al., 2006). Compared to liposomes, SLN show several advantages such as excellent tolerability, good physical stability, protection of labile drugs, high drug payload, controlled drug release, low cost and ease of production (Müller et al., 2000; Mehnert and Mäder, 2001; Wissing et al., 2004; Sapino et al., 2005; Iscan et al., 2005).

For cosmetic purposes SLN exhibit an occlusive effect, which improves skin hydration and protection against sun exposure (Wissing and Müller, 2002; Üner et al., 2005). As these carriers could be applied to a damaged skin, possible cytotoxicity of these preparations should be evaluated.

Surfactants represent one of the most common constituents in topical pharmaceutical and cosmetic applications or cleansers. Moreover, in order to formulate stable submicron emulsions and SLN common additives such as lecithin, cholates, sorbates, polysorbates, poloxamers and stearylamine are employed (Cavalli et al., 1996; Mbela et al., 1998; Schöler et al., 2001; Heydenreich et al., 2003; Üner et al., 2004; Wissing et al., 2004; Schubert and Müller-Goymann, 2005).

To ensure that the topical preparations are innocuous, it is necessary to study the potential irritation of surfactantia or co-surfactantia by determining their toxicity. Both in vitro and in vivo data suggest cationic surfactantia to be the most detrimental, followed by anionic surfactantia and the least problematic being non-ionic surfactantia (Harvell et al., 1994; Korting et al., 1994; Wilhelm et al., 2001). With respect to the use of amphiphilic phospholipids alone or in combination with co-surfactant sodium cholate for SLN preparations, a slight cytotoxicity of peritoneal macrophages was reported by Schöler et al. (2001). In agreement with this, Schubert and Müller-Goymann (2005) reported an increase in cytotoxicity on human dermal fibroblast cells with increasing lecithin concentration in the SLNs. However, since the extend of SLN cytotoxicity was not far from that of the aqueous solution, they attributed the cytotoxicity

* Corresponding author. Tel.: +32 3 820 2716; fax: +32 3 820 2734.
E-mail address: annick.ludwig@ua.ac.be (A. Ludwig).

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of the SLNs mainly to the components of the aqueous phase.

The aim of present study is to examine the influence of the (co)surfactants used on the physical properties of the carrier systems prepared and subsequently on the cytotoxicity towards different cell lines. Biologically relevant targets for skin irritants are human keratinocytes or dermal fibroblasts (Van de Sandt et al., 1999; Moreno, 2000; Wilhelm et al., 2001). The choice of mouse 3T3 fibroblasts and human HaCaT keratinocytes as model system has been reported in the past, and therefore seems justified (Benavides et al., 2004). Mouse J774 macrophages were used in this study since several researchers (Catelas et al., 1999; Schrijvers et al., 2004) have recorded that they are able to take up large particles up to 1 μm and more, giving us the opportunity to check for the influence of particle size on cell survival.

2. Materials and methods

2.1. Materials

Soybean oil, stearic acid, sodium taurocholate, stearylamine, Dulbecco's modified Eagles medium (DMEM) were supplied by Sigma–Aldrich Chemie (Bornem, B). Soybean lecithin (Lipoid S75) and phosphatidylserine were purchased from Lipoid GmbH (Ludwigshafen, D). Mannitol, isopropylalcohol, polysorbate 80 were purchased from VWR International (Leuven, B). Following semi-synthetic glycerides or hard fat (Ph. Eur.) Witepsol H12, H185, E75, E76, E85, S55, W45 were obtained from Sasol (Witten, D), Suppocire AS2 from Gattefossé (Lyon, F) and Novata C from Febelco (Antwerp, B)

3-(4,5-Dimethyl 2-thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT) was purchased from Sigma–Aldrich Chemie (Bornem, B). Cell lines used were J774A1 (European Collection of Cell Cultures ECACC) and 3T3 mouse BALB/c fibroblasts (American tissue culture collection ATCC). The HaCaT cell line was kindly provided by Prof. J. Merregaert (Laboratory of Molecular Biotechnology, University of Antwerp, Belgium). Microtiter plates were obtained from TPP (Trasadingen, CH)

Purified water produced by Milli-Q (Millipore Co., Bedford, USA) was used throughout the experiments. All reagents used were of pharmaceutical or analytical grade.

2.2. Preparation of submicron emulsion

Submicron emulsions were prepared by means of a phase inversion method. The required amount of lecithin (Lipoid S75) and cosurfactants (sodium taurocholate, phosphatidylserine, stearylamine) were heated (70 °C) and mixed together with soybean oil (8 g). Afterwards the heated aqueous phase (5.07% (w/v) mannitol) was added in a slow stream under stirring to the warm oil phase in order to obtain an O/W emulsion. The final weight of the emulsion was 40 g.

The primary emulsion obtained by mechanical mixing during 5 min was further homogenized with an ultrasonic probe (Branson Sonifier, B12, Danbury, USA) during 2 min at 35% power or 14 W amplitude. The emulsions were stored in a refrigerator at 5 °C.

2.3. Preparation of solid lipid nanoparticles (SLN)

Solid lipid nanoparticles were prepared by melt-emulsification. Firstly the surfactants (lecithin, sodium taurocholate, polysorbate 80, stearylamine) were mixed with stearic acid or hard fats, together with part of the aqueous mannitol (5.07%, w/v) solution and heated under stirring. Then the samples were sonicated (Branson Sonifier, Danbury, USA) at 80 °C. The emulsion obtained was cooled down to about 15 °C by pooring the second part of the mannitol solution, which was kept at 2 °C, in order to obtain solid nanoparticles. The total weight of each preparation was 60.0 g. The samples were stored in a refrigerator at 5 °C.

2.4. Physical characterization

Measurement of droplet size, polydispersity index and zetapotential were performed using the Zetasizer 3000 (Malvern Instruments, Malvern, UK). The mean droplet size Z_{ave} was measured by photon correlation spectroscopy (PCS) after appropriate dilution with the vehicle. Each sample was measured three times and the average value calculated. Zetapotential values of the samples were determined using electrophoretic light scattering (ELS). Each sample was diluted with vehicle, resulting in optimum signal intensity. The dispersion was injection in the capillary of the Zetasizer 3000, and measured 10 times. The measurements were performed in triplicate and mean values were calculated.

2.5. Cytotoxicity test

Cellular damage results in loss of the metabolic cell function. The tetrazolium salt MTT is widely used to quantitate by colorimetric assay the cytotoxicity of preparations. The tetrazolium salts are metabolically reduced to highly colored end products called formazans (Mosmann, 1983). The colorless MTT is cleaved to formazan by the succinate-tetrazolium reductase system which belongs to the mitochondrial respiratory chain and is active only in viable cells (Hoper, 1997).

Ninety-six well microtiter plates were inoculated with 0.5×10^4 mouse 3T3 fibroblast cells, HaCaT keratinocyte cells or J774 macrophage cells in Dulbecco's modified Eagles medium (DMEM containing 10% bovine serum, 4 mM L-glutamine, 100 U/ml Penicillin 100 μg/ml streptomycin), hereafter referred to as growth medium, and cells were incubated at 37 °C overnight in 5% CO_2 atmosphere (CB serie, Binder Gmbh, D). After overnight incubation, nanoparticles or submicron emulsion sample were added to the medium and cells were incubated for another 18 h. The medium in the plates was discarded and wells were washed twice with PBS, then MTT in growth medium was added at a final concentration of 0.5 mg/ml.

After 2 h incubation, the insoluble formazan crystals were solubilzed with 0.04N HCl in isopropylalcohol and absorption was measured on a plate reader MRX-II (Dynex Technologies Inc., Chantilly, VA, USA) at 570 nm. Reference measurements were performed at 690 nm, where neither MTT nor formazan

Table 1
Composition of the microemulsions studied (soybean oil was used as matrix)

Code	Surfactants
SMEM 1	Lipoid S75 0.8 g
SMEM 2	Lipoid S75 0.6 g
SMEM 3	Lipoid S75 0.4 g
SMEM 4	Lipoid S75 0.8 g + taurocholate (T) 0.08 g
SMEM 5	Lipoid S75 0.8 g + phosphatidylserine (PS) 0.08 g
SMEM 6	Lipoid S75 0.8 g + taurocholate 0.08 g + PS 0.08 g
SMEM 7	Lipoid S75 0.8 g + taurocholate 0.08 g + stearylamine (SA) 0.08 g
SMEM 8	Lipoid S75 0.8g + T 0.08 g + PS 0.08 g + SA 0.08 g
SMEM 9	Lipoid S75 0.4 g + taurocholate 0.04 g
SMEM 10	Lipoid S75 0.4 g + phosphatidylserine 0.04 g
SMEM 11	Lipoid S75 0.4 g + taurocholate 0.04 g + PS 0.04 g

absorb, in order to eliminate errors from scratches on the plastic well or turbidity. For each of the samples evaluated, the test was performed in triplicate and reference cells were treated with milliQ water.

2.6. Statistics

Each study was performed in triplicate and the data were expressed as mean ± standard deviation (S.D.). A two-tailed Student's *t*-test was performed on the data compared to control.

3. Results and discussion

3.1. Submicron emulsions

Submicron emulsions were prepared with different kinds of lecithin and cosurfactants in order to obtain emulsion droplets of different charges. The composition of the various emulsions is summarised in Table 1.

3.1.1. Physical characterisation

The physical characteristics of all submicron emulsions were measured 1 day after preparation and storage at 5 °C. After preliminary studies, it was decided to employ following sonication parameters: 2 min and 35% power. The data obtained are given in Table 2.

Table 2
Physical characteristics of submicron emulsions ($n = 3$)

Preparations	Droplet size (nm) (±S.D.)	Zetapotential (mV) (±S.D.)
SMEM 1	767.5 (96.7)	−31 (1.1)
SMEM 2	808.9 (56.7)	−30 (0.6)
SMEM 3	900.4 (54.0)	−32 (0.9)
SMEM 4	545.5 (41.2)	−33 (1.4)
SMEM 5	574.6 (7.4)	−39 (0.4)
SMEM 6	538.5 (3.6)	−34 (0.9)
SMEM 7	598.6 (25.4)	−28 (3.3)
SMEM 8	538.3 (89.5)	−27 (1.2)
SMEM 9	603.5 (37.5)	−33 (1.1)
SMEM 10	697.6 (117.7)	−29 (2.7)
SMEM 11	596.3 (22.6)	−31 (0.5)

The mean droplet size measured ranged from 538 nm to 900 nm. The largest droplets were determined when submicron emulsions were formulated with the surfactant Lipoid S75 only. As expected, the higher the concentration of lecithin the smaller the droplet size measured. Enough surfactant molecules are present to form a strong monolayer at the interface of small oil droplets and to stabilize the submicron emulsion (Mbela et al., 1998).

The addition of cosurfactants had a pronounced effect on the droplet size. The lowest droplets sizes were measured when a blend of sodium taurocholate and phosphatidylserine with and without stearylamine was added to lecithin. Incorporation of stearylamine on formulation SMEM 4 resulted, however, in a slight size increase.

In general, the polydispersity indices of the submicron emulsions formulated with cosurfactants were smaller than in the case of lecithin Lipoid S75.

The various carrier systems prepared were negatively charged. The addition of the cationic stearylamine did not result in positive charged oil droplets, but only a reduction of the zetapotential value from −33 mV to −27 mV was observed. Due to the blend of surfactant and cosurfactants present, the stearylamine molecule could probably not reach the interface in the required position in order to obtain positively charged oil droplets.

If from a therapeutical point of view small droplets are required, changes of the homogenisation parameters allow achieving this goal. Increasing the sonication time from 2 min to 5 min at 20% power or 8 W amplitude resulted in the case of SMEM 1 and SMEM 8 in a size decrease from 954 nm to 662 nm and from 600 nm to 442 nm, respectively. Also a decrease of the polydispersity indices was noted.

The duration of the sonication had a smaller effect on the droplet size than the power employed during homogenisation. After homogenisation at a power of 50% (20 W amplitude) during 5 min submicron emulsions with droplet sizes of 314 nm (SMEM 1) and 363 nm (SMEM 8) were obtained.

3.1.2. Cytotoxicity

The results of the cytotoxicity test performed in present study on different cell lines point to the importance of the surfactant/cosurfactant used to prepare the emulsions (Fig. 1 and Table 3). When the cytotoxicity of different (co)surfactants in submicron emulsions of soybean oil was compared to control, it was noticed that only the addition of stearylamine was toxic to the three cell lines investigated. The HaCaT cells were the least affected and resisted best to stearylamine. The results observed were in agreement with the reported apoptosis induction of cationic liposomes in different cell lines (Aramaki et al., 2002; Takano et al., 2003). However, this toxicity has not yet been reported for submicron emulsions with stearylamine as (co)surfactant. In contrast to cationic liposomes, the submicron emulsions SMEM 7 and 8 did not possess a net positive surface charge. The presence of stearylamine at the interface of the oil droplets or free in the emulsion induced cytotoxicity. The concentration of the submicron emulsion used in the test had an influence on the viability of the cells in contact with the prepa-

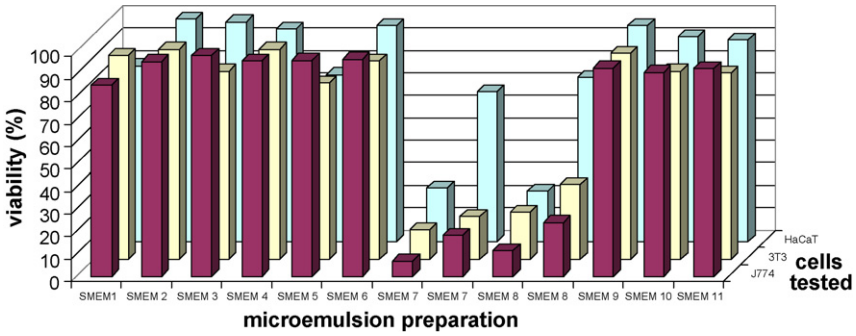

Fig. 1. Cytotoxicity of microemulsions in three cell lines (expressed as % viability) ($n = 3$).

rations. A concentration of 1% is less toxic compared to 5%. A viability % increase from 1.5 to 3 times was observed in the case of SMEM 7 and SMEM 8.

The (co)surfactants lecithin, phosphatidylserine and sodium taurocholate did not cause significant cytotoxicity. The amount (0.04 g or 0.08 g) of cosurfactant present in the formulation did not influence the viability of the three cell lines examined.

3.2. SLN

Four main preparation methods for SLN are reported in literature: (i) high shear homogenization and/or ultrasound; (ii) high-pressure homogenization including the hot and cold homogenization technique; (iii) solvent emulsification/evaporation; (iv) via microemulsion (Üner, 2006). The most popular preparation method involves the use of high-pressure homogenisation to size down the particles to the submicron range. In both the hot and the cold method, the lipid is first melted in order to incorporate the drug into the matrix. In the present study the ultrasound method was selected, because the same power (35%) could be used as for the preparation of the submicron emulsions.

High shear homogenization and ultrasound method share the problem that the SLNs obtained generally have a broad particle size distribution, leading to physical instabilities such as particle growth during storage. Particle size distribution could be improved by higher surfactant concentrations, but the correspondent toxicological implications must be evaluated.

In order to investigate the influence of the type and amount of surfactant used to prepare solid nanoparticles on the physical properties and cytotoxicity, various formulations were prepared. The composition is summarised in Table 4.

The influence of the core material on the properties of nanoparticles was examined by preparing SLNs using various kind of semi-synthetic glycerides or hard fat (Eur. Ph.). According to the European Pharmacopea hard fat consists of a mixture of triglycerides, diglycerides and monoglycerides, which may be obtained either by esterification of fatty acids of natural origin with glycerol or by transesterification of natural fats. Each type of hard fat is characterised by its melting point, its hydroxyl value and its saponification value. The physicochemical properties of the hard fats used are given in Table 5.

3.2.1. Physical characterisation

Solid lipid nanoparticles made of stearic acid as core material were stabilized with the Surfactant Lipoid S75, sodium taurocholate, polysorbate 80, stearylamine or blends.

It was not possible to prepare stable nanoparticles when using only Lipoid S75 or stearylamine in the concentration selected. As can be deduced from Table 6, the nanoparticles prepared had a size ranging from 280 nm to 380 nm, meaning that differences in particle size were not very significant. Increasing the amount

Table 3
Cytotoxicity of microemulsions in three cell lines (expressed as % viability) (±S.D.) ($n = 3$)

Preparation	Concentration (%)	J774	3T3	HaCaT
Control	0	100 (1.60)	100 (4.31)	100 (12.15)
SMEM 1	5	85.21 (7.40)	90.94 (9.91)	79.42 (5.75)
SMEM 3	5	98.27 (0.36)	83.62 (6.59)	98.06 (13.18)
SMEM 4	5	95.88 (5.80)	93.43 (12.18)	94.95 (8.14)
SMEM 5	5	96.16 (3.31)	78.45 (4.44)	74.49 (13.80)
SMEM 6	5	96.74 (2.50)	88.44 (7.40)	96.72 (10.03)
SMEM 7	5	6.83 (2.44)*	13.14 (3.45)*	24.34 (4.61)
SMEM 7	1	18.47 (3.12)*	18.87 (6.12)*	66.94 (4.82)
SMEM 8	5	11.46 (3.01)*	21.18 (2.81)*	22.70 (1.57)*
SMEM 8	1	24.02 (2.68)*	33.30 (12.91)*	73.37 (1.39)
SMEM 9	5	92.72 (10.14)	91.77 (9.72)	96.33 (13.21)
SMEM 10	5	90.86 (9.64)	83.63 (5.95)	93.14 (2.98)
SMEM 11	5	92.27 (10.05)	82.70 (2.42)	89.90 (16.92)

* $p < 0.01$ compared to control.

Table 4
Composition of solid lipid nanoparticles studied

Code	Core material	Surfactants
SLN 1	Stearic acid 0.2 g	Lipoid S75 0.10 g + T 0.10 g
SLN 2	Stearic acid 0.2 g	Lipoid S75 0.15 g + T 0.15 g
SLN 3	Stearic acid 0.2 g	Polysorbat 80 0.03 g
SLN 4	Stearic acid 0.2 g	Polysorbat 80 0.06 g
SLN 5	Stearic acid 0.2 g	P80 0.03 g + T 0.05 g
SLN 6	Stearic acid 0.2 g	P80 0.06 g + T 0.05 g
SLN 7	Stearic acid 0.2 g	P80 0.03 g + S75 0.05 g
SLN 8	Stearic acid 0.2 g	P80 0.03 g + S75 0.05 g + T 0.05 g
SLN 9	Stearic acid 0.2 g	P80 0.03 g + SA 0.04 g
SLN 10	Stearic acid 0.2 g	P80 0.03 g + SA 0.05 g
SLN 11	Stearic acid 0.2 g	P80 0.03 g + SA 0.08 g
SLN 12	Suppocire AS2 0.2 g	Lipoid S75 0.15 g + T 0.15 g
SLN 13	Novata C 0.2 g	Lipoid S75 0.15 g + T 0.15 g
SLN 14	Witepsol E75 0.2 g	Lipoid S75 0.15 g + T 0.15 g
SLN 15	Witepsol E76 0.2 g	Lipoid S75 0.15 g + T 0.15 g
SLN 16	Witepsol E85 0.2 g	Lipoid S75 0.15 g + T 0.15 g
SLN 17	Witepsol H12 0.2 g	Lipoid S75 0.15 g + T 0.15 g
SLN 18	Witepsol H182 0.2 g	Lipoid S75 0.15 g + T 0.15 g
SLN 19	Witepsol S55 0.2 g	Lipoid S75 0.15 g + T 0.15 g
SLN 20	Witepsol W45 0.2 g	Lipoid S75 0.15 g + T 0.15 g

of polysorbate 80 resulted not only in smaller nanoparticles but also in a smaller polydispersity index (<0.1), indicating that the samples were monomodal. An increase of the amount of stearylamine, however, led to the formation of larger particles. Sodium taurocholate had a small influence on the particle size. In general one could conclude that the size of the nanoparticles is not strongly depended on the type of surfactant or blends of surfactants selected. There was no significant difference in size of the various stearic acid nanoparticles manufactured.

Addition of stearylamine did not lead to positively charged nanoparticles, but as in the case of submicron emulsion only a decrease of the zetapotential was measured. Nanoparticles stabilized with the non-ionic surfactant polysorbate 80 were also negatively charged.

The results of the particle size and zetapotential measurements of the nanoparticles formulated with different kinds of hard fat but the same surfactant blend Lipoid S75 and sodium taurocholate are given in Table 7.

The smallest nanoparticles were measured when the core material possesses a high hydroxyl value (Witepsol S55). This was expected, because a high hydroxyl value means a high amount of mono- and diglycerides and thus high emulsification properties.

In the series of Witepsol E there was no linear relationship between particle size and hydroxyl value, but Witepsol E75 having the highest acid value showed the smallest particle size (347 nm instead of 380 nm and 391 nm).

Witepsol S55 nanoparticles showed the smallest zetapotential value, but no linear relationship between zetapotential and chemical properties of the core material could be observed. All zetapotential values were in the range −20 mV to −30 mV, which means about the critical value for electrostatic repulsion of colloidal particles, but good physical stability of the preparations was observed probably due to a steric effect of the surfactants.

Table 6
Physical characteristics of stearic acid solid lipid nanoparticles ($n = 3$)

Preparation	Particle size (nm) (±S.D.)	Zetapotential (mV) (±S.D.)
SLN 1	348.1 (66.1)	−31.2 (1.3)
SLN 2	353.7 (31.8)	−27.6 (0.4)
SLN 3	366.7 (11.3)	−25.3 (1.3)
SLN 4	283.7 (2.5)	−27.1 (5.6)
SLN 5	319.6 (3.5)	−31.9 (1.8)
SLN 6	291.8 (6.5)	−28.7 (0.6)
SLN 7	342.0 (3.2)	−25.9 (0.5)
SLN 8	338.7 (2.7)	−27.2 (0.3)
SLN 9	283.7 (10.8)	−22.4 (1.0)
SLN 10	357.5 (5.6)	−23.2 (0.3)
SLN 11	381.5 (12.5)	−18.0 (1.4)

Table 7
Physical characteristics of hard fat solid lipid nanoparticles ($n = 3$)

Preparation	Particle size (nm) (±S.D.)	Zetapotential (mV) (±S.D.)
SLN 12 Suppocire AS2	310.0 (3.4)	−2.0 (1.5)
SLN 13 Novata C	413.2 (50.7)	−26.7 (1.4)
SLN 14 Witepsol E75	347.5 (3.0)	−32.8 (0.9)
SLN 15 Witepsol E76	391.4 (17.7)	−29.5 (1.0)
SLN 16 Witepsol E85	380.6 (7.8)	−22.2 (1.3)
SLN 17 Witepsol H12	327.1 (14.7)	−29.6 (1.7)
SLN 18 Witepsol H185	268.0 (10.3)	−25.6 (1.0)
SLN 19 Witepsol S55	255.6 (5.0)	−25.5 (2.5)
SLN 20 Witepsol W45	429.3 (11.7)	−27.7 (0.9)

Table 5
Physico-chemical properties of semi-synthetic glycerides or hard fat used

Hard fat	mp (°C)	Hydroxyl value	Saponification value	Max acid value
Suppocire AS2	35	15–25	224–246	0.5
Novata C	36	20–30	225–235	0.3
Witepsol E75	37	15	220–230	1.3
Witepsol E76	37	30–40	220–230	0.3
Witepsol E85	42	15	220–230	0.3
Witepsol H12	32	15	240–255	0.2
Witepsol H185	39	15	220–235	0.2
Witepsol S55	33.5	50–65	215–230	1
Witepsol W45	33.5	40–50	225–235	0.3

mp = melting point. Hydroxyl value is the number of mg KOH needed to neutralize the amount of acetic acid used to acetylating 1 g of fat. Saponification value is the amount of KOH in mg required to saponify 1 g of fat. Acid value is the amount of KOH in mg required to neutralize the organic acids contained in 1 g of fat.

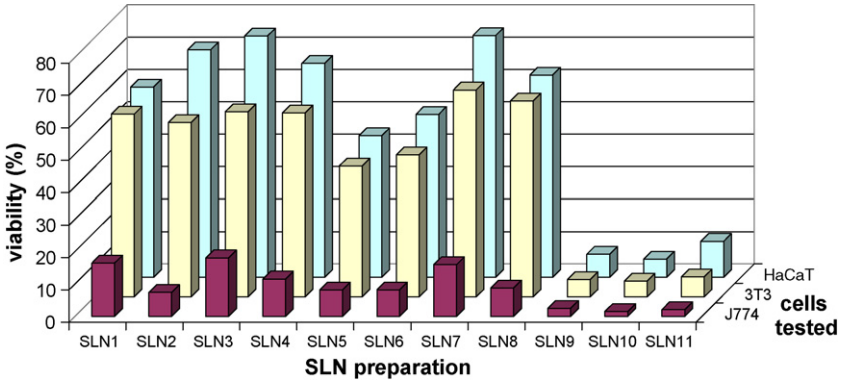

Fig. 2. Cytotoxicity of stearic acid solid lipid nanoparticles in three cell lines (expressed as % viability) ($n = 3$).

Table 8
Cytotoxicity of stearic acid solid lipid nanoparticles in three cell lines (expressed as % viability) (±S.D.) ($n = 3$)

Preparation	J774	3T3	HaCaT
Control	100 (8.92)	100 (15.49)	100 (3.47)
SLN 1	16.54 (1.43)*	56.39 (3.26)§	58.79 (5.36)*
SLN 2	7.40 (0.66)*	53.83 (2.89)§	70.26 (6.32)*
SLN 3	18.13 (2.63)*	57.19 (3.26)§	74.55 (6.77)§
SLN 4	11.60 (2.61)*	56.87 (5.03)§	66.16 (4.90)*
SLN 5	8.19 (2.09)*	40.42 (2.77)§	43.71 (5.35)*
SLN 6	8.27 (1.15)*	43.77 (4.55)§	50.35 (9.67)*
SLN 7	16.02 (3.37)*	63.90 (1.69)§	74.66 (5.24)§
SLN 8	8.77 (1.76)*	60.54 (6.24)§	62.49 (4.97)§
SLN 9	2.47 (0.45)*	5.43 (1.00)*	7.19 (2.23)*
SLN 10	1.45 (0.45)*	4.95 (1.47)*	5.68 (1.82)*
SLN 11	2.10 (0.50)*	6.23 (1.66)*	11.18 (1.94)*

* $p < 0.01$ compared to control.
§ $p < 0.05$ compared to control.

3.2.2. Cytotoxicity

Due to the presence of stearylamine in stearic acid nanoparticles the cytotoxicity test was performed at a concentration of 1%. The results are presented in Fig. 2 and Table 8.

The nanoparticles formulated with the fatty acid stearic acid were cytotoxic for all cell lines. However, macrophages were most affected. This could be possibly accounted to the fact that the main function of the macrophages is capturing foreign particles entering the human body. Considering the fact that the particle size of all nanoparticles being smaller than 500 nm, phagocytosis combined with pinocytosis by macrophages could occur (Aukunuru and Kompella, 2002; Chellat et al., 2005). It is therefore not unlikely that the amount of nanoparticles taken up per cell is much higher in macrophages compared to the other cells. Secondly, since cell types differ profoundly from each other in metabolic activity and ability to deal with e.g. oxidative stress (Clothier et al., 2002), the toxic effects of the samples differ. It has been shown that both stearic acid and stearylamine induce apoptosis (Iwaoka et al., 2006; Lu et al., 2003; Martins de Lima et al., 2006).

The results obtained are in agreement with the work of Schöler et al. They demonstrated the impact of surfactants used to prepare solid lipid nanoparticles on the viability of macrophages. A marked toxicity was measured when nanoparticles were coated with the cationic surfactant cetylpyridinium chloride, while formulations containing Lipoid S75 reduced cell viability only slightly (Schöler et al., 2001).

In order to compare the influence of the core material on cell survival, the cytotoxicity test with hard fat nanoparticles was carried out at a concentration of 5%. The results obtained are reported in Fig. 3 and Table 9.

The different hard fat matrices did not affect the viability of the three cell lines. Cell survival was only slighly influenced with the exception of SLN19, where a statistical difference was noticed for J774 cells. This could possibly be ascribed to the sensibility of this cell type for higher hydroxylation value of semi-synthetic glycerides, although future investigation on the subject is needed. Viability between 76% and 99% was measured. The use of semi-synthetic glycerides seems more appropriate than stearic acid. The choice will depend on the

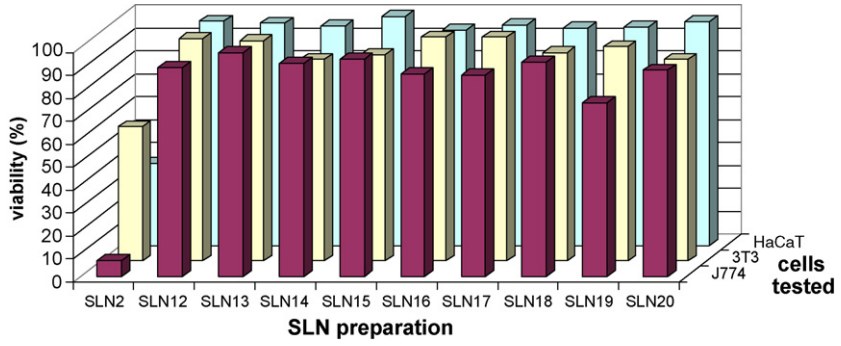

Fig. 3. Cytotoxicity of hard fat solid lipid nanoparticles in three cell lines (expressed as % viability).

Table 9
Cytotoxicity of hard fat solid lipid nanoparticles in three cell lines (expressed as % viability) (±S.D.)

Preparation	J774	3T3	HaCaT
Control	100 (8.92)	100 (11.77)	100 (3.47)
SLN 12	91.37 (5.15)	96.83 (13.67)	97.76 (4.53)
SLN 13	97.68 (2.95)	96.00 (11.81)	96.92 (8.44)
SLN 14	93.19 (7.51)	87.88 (3.52)	95.71 (7.81)
SLN 15	94.92 (8.99)	89.94 (2.69)	99.81 (2.52)
SLN 16	88.54 (4.65)	97.66 (2.52)	93.66 (3.86)
SLN 17	88.18 (2.33)	97.66 (10.70)	96.15 (3.94)
SLN 18	93.76 (5.59)	90.77 (5.73)	94.57 (2.46)
SLN 19	75.92 (7.94)*	93.53 (10.26)	95.34 (2.64)
SLN 20	90.14 (8.21)	87.88 (4.93)	97.62 (4.39)

* $p < 0.01$ compared to control.

physicochemical properties of the drugs to be incorporated and the drug release rate required for the therapeutical effect considered.

4. Conlcusion

Submicron O/W emulsions and nanoparticles formulated with lecithin, sodium taurocholate, phosphatidylserine and polysorbate 80 did not affect the viability of mouse fibroblast cells, HaCaT keratinocyte cells or J774 macrophage cells. Cell survival was highly affected by using stearylamine.

Nanoparticles made of stearic acid are cytotoxic, especially for J774 macrophages and to a lower extent for mouse 3T3 fibroblasts and HaCaT keratinocytes. In general a viability of more than 90% was observed when semi-synthetic glycerides were used to manufacture nanoparticles.

The choice of the lipid matrix and the surfactant is essential in order to formulate a safe and stable preparation.

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References

Aramaki, Y., Takano, S., Arima, H., Tsuchiya, S., 2002. Induction of apoptosis in WEHI 231 cells by cationic liposomes. Pharm. Res. 17, 515–520.

Aukunuru, J., Kompella, U., 2002. In vitro delivery of nano- and microparticles to human retina epithelial (ARPE-19) cells. Drug Del. Technol. 2, 50–57.

Benavides, T., Mitjans, M., Martinez, V., Clapes, P., Infante, M., Clothier, R., Vinardell, M., 2004. Assessment of primary eye and skin irritants by in vitro cytotoxicity and phototoxicity models: an in vitro approach of new arginine-base surfactant-induced irritation. Toxicology 197, 229–237.

Catelas, I., Petit, A., Zukor, D.J., Marchand, R., Yahia, L., Huk, O.L., 1999. Induction of macrophage apoptosis by ceramic and polyethylene particles in vitro. Biomaterials 20, 625–630.

Cavalli, R., Marengo, E., Rodriguez, L., Gasco, M.R., 1996. Effects of some experimental factors on the production process of solid lipid nanoparticles. Eur. J. Pharm. Biopharm. 43, 110–115.

Cevc, G., 2004. Lipid vesicles and other colloids as drug carriers on the skin. Adv. Drug Del. Rev. 56, 675–711.

Chellat, F., Mehri, Y., Moreau, A., Yahia, L., 2005. Therapeutic potential of nanoparticulate systems for macrophage targeting. Biomaterials 26, 7260–7275.

Chen, H., Chang, X., Du, D., Liu, W., Liu, J., Weng, T., Yang, Y., Xu, H., Yang, X., 2006. Podophyllotoxin-loaded solid lipid nanoparticles for epidermal targeting. J. Contr. Rel. 110, 296–306.

Clothier, R., Starzec, G., Pradel, L., Baxter, V., Jones, M., Cox, H., Noble, L., 2002. The prediction of human skin responses by using the combined in vitro fluorescein leakage/Alamar Blue (resazurin) assay. Altern. Lab. Anim. 30, 493–504.

Harvell, J., Tsai, Y.C., Maibach, H.I., Gay, R., Gordon, V.C., Miller, K., Mun, G.C., 1994. An in vivo correlation with three in vitro assays to assess skin irritation potential. J. Toxicol. Cutan. Ocular. Toxicol. 13, 171–183.

Hoper, J., 1997. Spectrophotometric in vivo determination of local mitochondrial metabolism by use of a tetrazolium salt. Physiol. Meas. 18, 61–66.

Heydenreich, A.V., Westmeier, R., Pedersen, N., Poulsen, H.S., Kristensen, H.G., 2003. Preparation and purification of cationic solid lipid nanospheres—effects on particle size, physical stability and cell toxicity. Int. J. Pharm. 254, 83–87.

Iscan, Y., Wissing, S.A., Hekimoglu, S., Müller, R.H., 2005. Solid lipid nanoparticles (SLN™) for topical drug delivery: incorporation of the lipophilic drugs *n*,*N*-diethyl-*m*-toluamide and vitamin K. Pharmazie 60, 905–909.

Iwaoka, S., Nakamura, T., Takano, S., Tsuchiya, S., Aramaki, Y., 2006. Cationic liposomes induce apoptosis through p38 MAP kinase-caspase-8-Bid pathway in macrophage-like RAW264.7 cells. J. Leukoc. Biol. 79, 184–191.

Korting, H.C., Herzinger, T., Hartinger, A., Kerscher, M., Angerpointner, T., Maibach, H.I., 1994. Discrimination of the irritancy potential of surfactants in vitro by two cytotoxicity assays using normal human keratinocytes, HaCaT cells and 3T3 mouse fibroblasts: correlation with in vivo data from a soap chamber assay. J. Dermatol. Sci. 7, 119–129.

Lombardi Borgia, S., Regehly, M., Sivaramakrishnan, R., Mehnert, W., Korting, H.C., Danker, K., Röder, B., Kramer, K.D., Schäfer-Korting, M., 2005. Lipid nanoparticles for skin penetration enhancement—correlation to drug localization within particle matrix as determined by fluorescence and parelectric spectroscopy. J. Contr. Rel. 110, 151–163.

Lu, Z.H., Mu, Y.M., Wang, B.A., Li, X.L., Lu, J.M., Li, J.Y., Pan, C.Y., Yanase, T., Nawata, H., 2003. Saturated free fatty acids, palmitic acid and stearic acid, induce apoptosis by stimulation of ceramide generation in rat testicular Leydig cell. Biochem. Biophys. Res. Commun. 303, 1002–1007.

Martins de Lima, T., Cury-Boaventura, M.F., Giannocco, G., Nunes, M.T., Curi, R., 2006. Comparative toxicity of fatty acids on a macrophage cell line (J774). Clin. Sci. (Lond.) 111, 307–317.

Mbela, T., Verschueren, E., Ludwig, A., 1998. Influence of stearylamine and dicetylphosphate on the physical properties of submicron O/W emulsions. J. Pharm. Belg. 53, 81–86.

Mehnert, W., Mäder, K., 2001. Solid lipid nanoparticles. Production, characterization and applications. Adv. Drug Del. Rev. 47, 165–196.

Moreno, J.J., 2000. Arachidonic acid release and prostaglandin E2 synthesis as irritant index of surfactants in 3T6 fibroblast cultures. Toxicology 143, 275–282.

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Meth. 65, 55–63.

Müller, R.H., Mäder, K., Gohla, S., 2000. Solid lipid nanoparticles (SLN) for controlled drug delivery—a review of the state of the art. Eur. J. Pharm. Biopharm. 50, 161–177.

Müller, R.H., Radtke, M., Wissing, S.A., 2002. Solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC) in cosmetic and dermatological preparations. Adv. Drug Del. Rev. 54, S131–S155.

Sapino, S., Carlotti, M.E., Pelizzetti, E., Vione, D., Trotta, M., Battaglia, L., 2005. Protective effect of SLNs encapsulation on the photodegradation and thermal degradation of retinyl palmitate introduced in hydroxyethylcellulose gel. J. Drug Del. Sci. Technol. 15, 159–165.

Schöler, N., Olbrich, C., Tabatt, K., Müller, R.H., Hahn, H., Liesenfeld, O., 2001. Surfactant, but not the size of solid lipid nanoparticles (SLN) influences viability and cytokine production of macrophages. Int. J. Pharm. 221, 57–67.

Schrijvers, D.M., Martinet, W., De Meyer, G.R., Andries, L., Herman, A.G., Kockx, M.M., 2004. Flow cytometric evaluation of a model for phagocytosis of cells undergoing apoptosis. J. Immunol. Meth. 287, 101–108.

Schubert, M.A., Müller-Goymann, C.C., 2005. Characterisation of surface-modified solid lipid nanoparticles (SLN): influence of lecithin and nonionic emulsifier. Eur. J. Pharm. Biopharm. 61, 77–86.

Takano, S., Aramaki, Y., Tsuchiya, S., 2003. Physicochemical properties of liposomes affecting apoptosis induced by cationic liposomes in macrophages. Pharm. Res. 20, 962–968.

Üner, M., Wissing, S.A., Yener, G., Müller, R.H., 2004. Influence of surfactants on the physical stability of solid lipid nanoparticle (SLN) formulations. Pharmazie 59, 331–332.

Üner, M., Wissing, S.A., Yener, G., Müller, R.H., 2005. Skin moisturing effect and skin penetration of ascorbyl palmitate entrapped in solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC) incorporated into hydrogel. Pharmazie 60, 751–755.

Üner, M., 2006. Preparation, characterization and physico-chemical properties of solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC): their benefits as colloidal drug carrier systems. Pharmazie 61, 375–386.

Van de Sandt, J., Roguet, R., Cohen, C., Esdaile, D., Panec, M., Corsini, E., Parker, C., Fusenig, N., Liebsch, M., Benford, D., de Brugerolle de Fraissinette, A., Fortach, M., 1999. The use of human keratinocytes and human skin models for predicting skin irritation. Altern. Lab. Anim. 27, 723–743.

Wilhelm, K.P., Bottjer, B., Siegers, C.P., 2001. Quantitative assessment of primary skin irritants in vitro in a cytotoxicity model: comparison with in vivo human irritation tests. Br. J. Dermatol. 145, 709–715.

Wissing, S.A., Müller, R.H., 2002. The influence of the crystallinity of lipid nanoparticles on their occlusive properties. Int. J. Pharm. 242, 377–379.

Wissing, S.A., Kayser, O., Müller, R.H., 2004. Solid lipid nanoparticles for parenteral drug delivery. Adv. Drug Del. Rev. 56, 1257–1272.