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Research paper

The physical state of lipid nanoparticles influences their effect on *in vitro* cell viability

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ABSTRACT

Although lipid nanoparticles represent potent drug carriers, for many formulations toxicity data are rare. Thus, in this study, the effect of different lipid nanoparticles on the cell viability of L929 mouse fibroblasts was systematically investigated using the MTT assay. The formulations were composed of trimyristin, tristearin or cholesteryl myristate stabilized with poloxamer 188, polysorbate 80, polyvinyl alcohol or a blend of soybean phospholipid and sodium glycocholate. Depending on lipid and storage conditions, the nanoparticles were prepared in different physical states or crystal modifications leading to different particle shapes. The cell viability was influenced considerably by the physical state of the particle matrix with crystalline nanoparticles causing a stronger decrease in viability than the corresponding liquid or liquid crystalline particles. Effects on the cell viability were also related to the type of matrix lipid, stabilizer and the particle shape. However, the effects of differently shaped particles of different polymorphic modifications of crystalline tristearin were comparable. The low viability caused by poloxamer 188-stabilized particles could be correlated with a strong cell uptake which was investigated by confocal laser scanning microscopy.

1. Introduction

Various nanoparticulate formulations are under investigation for their potential as parenteral drug carrier and delivery systems [1–5]. Lipid nanoparticle (LNP) formulations like parenteral fat emulsions or liposomes have been used for this purpose for many years. However, there is a broad variety of other LNP formulations also featuring typical advantages like being composed of mostly physiological lipids, biodegradability and relatively simple production methods [4,5]. Among these, solid lipid nanoparticles, emulsions of supercooled melts or supercooled smectic particles are promising candidates. The physical state of these particles depends on the nature of the matrix lipids and on the preparation and storage conditions [4,6–9]. For example, after preparation by melt-homogenization, nanoparticles from the solid triglyceride trimyristin form supercooled liquid emulsion droplets which are stable when stored at room temperature. Only storage at lower temperatures (e.g. in a refrigerator) leads to trimyristin crystallization and thus to the formation of solid particles [7]. On the other hand, certain cholesterol esters like cholesteryl myristate form supercooled smectic nanoparticles (a thermotropic liquid crystalline state) at room temperature [9]. Also in this case, refrigeration leads to the crystallization of the matrix lipid. Thus, by simply changing the storage conditions, two different LNP formulations can be obtained from the same starting formulation. On crystallization, not only the physical state of the matrix lipid is altered but also the particle shape. The spherical triglyceride emulsion droplets and also the barrel-shaped liquid crystalline cholesterol ester particles transform to more or less anisometric, platelet-like crystalline particles [7,9–11]. Differently shaped particles can also be prepared without alteration of the physical state as has been shown for tristearin particles. Spherical solid particles can be formed when this lipid is in the crystalline α-modification, whereas tristearin in the crystalline β-modification forms anisometric particles [12,13].

Although LNP formulations are more or less extensively investigated, e.g. concerning their physicochemical properties, data on the *in vitro* or *in vivo* toxicity are either difficult to find or hardly comparable between different studies, especially regarding the

Abbreviations: TM, trimyristin (Dynasan® 114); TS, tristearin (Dynasan® 118); CM, cholesteryl myristate; F68, poloxamer 188 (Lutrol® F 68); Tw80, polysorbate 80 (Tween® 80); S100/SGC, blend of soybean phospholipid (Lipoid® S100) and sodium glycocholate; PVA, polyvinyl alcohol (Mowiol® 3-83); DiI, $DiIC_{18}(3)$; L, liquid; LC, liquid crystalline; C, crystalline; Cα, crystalline (α-modification); Cβ, crystalline (β-modification); PI, polydispersity index; LNPs, lipid nanoparticles.

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drug-free, plain carrier. This fact is mostly related to the huge number of formulations varying in composition or preparation method. Also, the test methods and types of cells under investigation differ. In addition, many tested formulations are carrying drugs precluding the evaluation of the effect of the unloaded carrier particles. Hitherto, the toxicity of emulsions of supercooled melts has not been specifically studied. Although these dispersions contain emulsion droplets, their different composition compared with parenteral fat emulsions makes a comparison difficult. The only study dealing with the cell compatibility of supercooled smectic LNPs indicates only marginal effects on the *in vitro* cell viability [14]. In contrast, a lot of *in vitro* and also some *in vivo* toxicity data are available for solid LNPs. Compared with polymeric NPs, a much lower *in vitro* toxicity has been reported, and compared with a parenteral fat emulsion, the viability of the test cells was only slightly reduced [15,16]. These studies also indicated a dependence of toxic effects on the type of matrix lipid and stabilizer [17]. Lipid matrices composed of fatty acids and charged stabilizers were more toxic than triglyceride matrices and non-ionic surfactants [16,18–20]. Repeatedly administered intravenously into mice at high doses, solid LNPs showed reversible or no pathological effects [21].

Since for the formulations investigated in this study only few or no *in vitro* data on toxic effects have been reported, a systematic investigation concerning the effect of different parameters was the primary aim of this study. The effect of the formulations on the *in vitro* cell viability of L929 mouse connective tissue fibroblasts was studied with the MTT assay. The first parameter to be explored was the formulation composition. The LNPs were prepared from three different lipids (trimyristin (TM), tristearin (TS), cholesteryl myristate (CM)) and several stabilizers (poloxamer 188 (F68), polysorbate 80 (Tw80), polyvinyl alcohol (PVA) or a blend of soybean phospholipids and sodium glycocholate (S100/SGC)). Each formulation was prepared in two different physical states (TM and CM) or different crystalline modifications (TS), which was the second parameter to be explored. A direct comparison was possible for the supercooled liquid and the crystalline state of TM, the supercooled smectic and the crystalline state of CM, and the crystalline α- and β-modification of TS. Since both the physical state of TM and CM and the crystal modification of TS are closely related to a certain particle shape, this was the third parameter to be investigated. Finally, in order to evaluate the results of the MTT assay in more detail, TM formulations stabilized with F68 or Tw80 were labelled with DiI and their uptake into the cells was investigated by confocal laser scanning microscopy.

2. Materials and methods

2.1. Materials

Trimyristin (Dynasan® 114) and tristearin (Dynasan® 118) were a kind gift of Sasol Germany (D-Witten), and soybean phospholipid (Lipoid® S100) was provided by Lipoid (D-Ludwigshafen), poloxamer 188 (Lutrol® F-68) and polyvinyl alcohol (Mowiol® 3-83) by BASF (D-Ludwigshafen) and Clariant (D-Frankfurt/Main), respectively. Cholesteryl myristate, sodium glycocholate, MTT and DiI ($DiIC_{18}(3)$) were from Sigma–Aldrich Chemie (D-Taufkirchen), polysorbate 80 (Tween® 80), thiomersal and potassium iodide from Caesar & Loretz (D-Hilden), glycerol 85% from Fagron (D-Barsbüttel), bisbenzimide H33256 from Applichem (D-Darmstadt), iodine from Merck (D-Darmstadt), saccharose, boric acid and dimethylsulfoxide from Carl Roth (D-Karlsruhe). Solvents for HPLC were obtained from Fisher Scientific (D-Nidderau). Cell culture media and supplements were purchased from PAA Laboratories (A-Pasching) and cell culture plastic labware from Greiner Bio-One (D-Frickenhausen). All substances were used as received. Water was purified by reverse osmosis and subsequent distillation.

2.2. Methods

2.2.1. Preparation of lipid nanoparticles

The dispersions contained 5% lipid and 4% stabilizer (all concentrations w/w). For the stabilizer blend S100/SGC, a concentration of 3.2%/0.8% was applied. The aqueous phase was an isotonic glycerol solution (2.25%) in all cases. Matrix lipid and surfactant-containing aqueous phase (stirred over night) were heated above the melting temperature of the lipid (i.e. to 70 °C for TM, 80 °C for TS, 90 °C for CM). TM formulations to be used in cell uptake studies were labelled by adding the fluorescent probe DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate) to the lipid (1 mg DiI/g lipid). After lipid melting, the aqueous phase was added and the mixture was pre-homogenized in the heat (temperature see above) for 1 min at 24,000 rpm using an Ultra-Turrax (T8, Jahnke & Kunkel, D-Staufen). This crude emulsion was transferred to a hot Microfluidizer M-110S (Microfluidics, US-Newton) and homogenized for 3–5 min at 450–850 bar. All dispersions were filtered through 0.2-μm syringe filters into sterilized glass vials that were stored tightly closed. In order to avoid microbial contamination, any subsequent handling or sampling of the formulations was performed under laminar air flow with sterile equipment. One fraction of the TM and CM samples was stored at room temperature, where the liquid state ('L'; TM) or the liquid crystalline state ('LC'; CM) of the matrix lipid was retained due to supercooling [7,9]. The other fractions of the samples and the TS samples were stored at 2–8 °C, which led to lipid crystallization ('C'). In order to crystallize the matrix of CM-Tw80 particles, this formulation was stored in an ethanol cooling bath at 0 °C for seven days followed by storage at 2–8 °C.

2.2.1.1. Further treatment of F68 formulations. In order to adjust the concentration of free, not particle-bound surfactant F68, formulations were dialysed (100 kDa MWCO CE membrane, Spektrum Europe, NL-Breda) for 62 h in a ratio 1 + 99 against an isotonic glycerol solution containing 1.6% F68. The medium was changed once after 24 h. Due to the absence of toxic effects from unbound F68 (see Section 3) and in order to avoid the risk of lipid recrystallization or microbial contamination, DiI-labelled F68 formulations used for uptake studies were not dialysed. After determining the lipid content (see below), the samples were diluted to a final concentration of 4% lipid and 1.6% unbound F68 in the aqueous phase using the dialysis medium.

2.2.1.2. Further treatment of Tw80 formulations. The amount of unbound Tw80 in the aqueous phase of the particle formulations was determined by tensiometry after sample dilution in water below the critical micelle concentration of Tw80 (0.0014%) [22]. The surface tension was measured for 300 s at room temperature recording 100 measurement points (Processor Tensiometer K12 with a standard Pt–Ir-plate, Krüss, D-Hamburg). The last 25 data points of three measurements were averaged. The Tw80 concentration was calculated using a calibration curve obtained from diluted Tw80 solutions. After determining the lipid content (see below), the samples were diluted to a final concentration of 4% lipid and 1.6% unbound Tw80 in the aqueous phase using isotonic glycerol solutions containing defined amounts of Tw80, which were individually prepared for each LNP formulation.

2.2.1.3. Further treatment of S100/SGC formulations. The amount of free, unbound S100/SGC could not be determined by appropriate methods. After determining the lipid content (see below), the samples were diluted to a final concentration of 4% matrix lipid using an isotonic glycerol solution.

2.2.1.4. Further treatment of TS-PVA formulations. The crystalline TS was initially present in the α-modification ('Cα') [12,13]. In order to achieve a transition into the β-modification ('Cβ'), the sample was stored at increasing temperatures in a water bath (1 h 45 °C, 2 h 47 °C, 2 h 49 °C, 6 h 50 °C and finally 8 h 51 °C). The transition progress was investigated in a Pyris I calorimeter (see Supporting Information). During this process, a small fraction of macroscopically visible, larger particles appeared which was removed by filtration (5-μm syringe filter). One fraction of the sample was then stored in a refrigerator without further treatment (Cβ). The lipid of the other fraction was remolten (heating to 80 °C for 15 min in a water bath) and freshly crystallized by storage in a refrigerator (Cα). The existence of the respective crystalline modification was investigated by small- and wide-angle X-ray diffractometry (see Supporting Information).

The amount of unbound PVA was determined by separation of the aqueous phase of the formulations using ultracentrifugation (XL-80 ultracentrifuge, rotor SW 55 Ti, Beckman Coulter, US-Fullerton). In brief, 200 μl of the sample was diluted to 5 ml with water (Cβ) or a 20% saccharose solution (Cα). Centrifugation (2 h, 45,000 rpm, 4 °C) led to a pellet formation by the Cβ sample and to a cream layer on top of the tube by the Cα sample. 300 μl of the aqueous phase was then mixed with 1.5 ml boric acid (4%) and 300 μl of a iodine/potassium iodide solution (1.3%/2.5%). A green complex was formed by iodine and the hydroxyl groups of PVA in the presence of boric acid [23,24]. The mixtures were diluted to 5 ml with water, and the absorbance was measured at a wavelength of 690 nm. The results of nine samples were averaged. The PVA concentration was calculated using a calibration curve. For further experiments, the samples were diluted to a final lipid concentration of approximately 4% TS and 2.2% unbound PVA in the aqueous phase using isotonic glycerol solution (Cα) or a preset PVA solution (Cβ).

2.2.2. Reference samples

Lipofundin® MCT 20% (B. Braun Melsungen, D-Melsungen) was diluted to a lipid content of 4% using an isotonic glycerol solution, which, as for the LNPs, was the aqueous phase of all reference samples. Soybean phospholipid liposomes were prepared by dispersing 4% S100 in the aqueous phase (3 h of shaking) followed by extrusion for 21 times through a 100-nm polycarbonate membrane filter using a Liposofast extruder (Avestin Europe, D-Mannheim). Finally, the dispersion was sterile filtered (0.2 μm).

Solutions of the stabilizers F68, Tw80 or PVA (3.2%) were prepared and a dispersion of S100/SGC (2.56%/0.64%) in aqueous phase was homogenized (70 °C, 3 min, 450 bar, Microfluidizer M-110S). The concentrations corresponded to the total nominal stabilizer concentration in a dispersion of 4% lipid. A thiomersal stock solution (0.1%) was also prepared. All solutions/dispersions were sterile filtered (0.2 μm).

2.2.3. Particle characterization

Particle sizes were measured by photon correlation spectroscopy (PCS, Zetasizer Nano ZS, Malvern Instruments, UK-Worcestershire) after dilution in filtered, demineralized water at 25 °C and at a scattering angle of 173° (λ_{laser}: 633 nm, η_{water}: 0.89 mPa s, RI_{water}: 1.33, $RI_{particles}$: 1.45). The results are given as the *z*-average diameter (intensity weighted mean diameter assuming spherical particles) and the polydispersity index (PI, measure for the relative width of the particle size distribution). Three consecutive measurements of 5 min duration were averaged for each sample.

Particle stability in cell culture medium was also investigated using PCS. Cell culture medium (RPMI 1640 supplemented with 10% foetal calf serum and 4 mM L-glutamine) was filtered (0.2 μm) and pre-warmed to 37 °C. The particle dispersions were diluted in the warm medium (ratio of 1:10) and incubated at 37 °C. Directly after mixing, after 2 h and after 24 h, 10 μl of the mixture was diluted with 1 ml filtered, demineralized water and measured.

The concentration of the matrix lipids TM and CM was determined by HPLC (System Gold 126, Beckman Coulter, US-Fullerton) using a LiChrocart column packed with LiChrospher 100 RP 18 (Merck, D-Darmstadt) and evaporative light scattering detection (Varex MKIII ELSD, Alltech, D-Unterhaching). The dispersions were dissolved in acetonitrile–tetrahydrofurane 20:80 (v/v). Acetonitrile–tetrahydrofurane 55:45 (v/v; TM) or 50:50 (v/v; CM) was used as mobile phase with an isocratic flow-rate of 1 ml/min. Peaks were integrated and the calculated areas from six measurements were averaged. The lipid concentrations were calculated using calibration curves of the respective lipid. The concentration of the matrix lipid TS was not determined by HPLC, since this lipid was only poorly soluble in the solvent. Thus, for the respective formulations, the nominal lipid content of 5% was used for calculations.

For determination of the DiI concentration, 100 μl of the respective particle formulations was dissolved to 5 ml in acetonitrile–tetrahydrofurane 20:80 (v/v) and absorbance was measured at 551 nm. The amount of DiI was calculated from a calibration curve. Three measurements were averaged.

The physical state of the matrix lipids TM and CM was investigated in a Micro DSC III calorimeter (Setaram, F-Caluire). Approximately 300 mg of the samples was measured at a scan rate of 0.5 °C/min in the temperature range between 25 °C and 65 °C (TM) or 95 °C (CM). A vessel filled with undecane was used as reference. DSC measurements of TS formulations are described in the Supporting Information.

The particle shape was investigated by cryo-transmission electron microscopy (Cryo-TEM). One droplet (6–7 μl) of the sample was applied to a copper grid covered by a holey carbon film (Quantifoil Micro Tools, D-Jena) in a wet chamber and excess of liquid was blotted automatically for three seconds between two strips of filter paper. Subsequently, the samples were rapidly plunged into liquid ethane (cooled to ~−180 °C) in a cryobox (Carl Zeiss NTS, D-Oberkochen). Excess of ethane was removed with a piece of filter paper. The samples were transferred with a cryo-transfer unit (Gatan 626-DH, US-Pleasanton) into the pre-cooled cryo-transmission electron microscope (Philips CM 120, NL-Eindhoven) operated at 120 kV and viewed under low-dose conditions. The images were recorded with a 1kK CCD camera (FastScan F114, TVIPS, D-Gauting).

2.2.4. MTT assay

The MTT assay was performed according to a modified method described by Mosmann [25] and Fischer et al. [26]. L929 mouse connective tissue fibroblasts (DSMZ, D-Braunschweig) were plated into 96-well tissue culture plates (25,000 cells/cm^2). The cells were grown for 24 h in RPMI 1640 medium supplemented with 10% foetal calf serum and 4 mM L-glutamine (37 °C, 5% CO_2, 95% relative humidity). The medium was removed and serial dilutions of LNPs (up to 0.5%), reference samples or control solutions (up to 1% corresponding lipid concentration) in culture medium were added. The thiomersal stock solution was diluted in culture medium (0.02%). Cell morphology was observed microscopically (Leica DMIL, Leica Microsystems, D-Wetzlar) and the osmolarity of highly concentrated sample dilutions was measured (Knauer semi-micro osmometer, D-Berlin). After 2 or 24 h, the supernatant was aspirated and the cells were washed with warm serum-free culture medium. A stock solution of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) in phosphate-buffered saline (5.5 mg/ml, pH 7.4) was diluted in serum-free culture medium to 0.5 mg/ml and added to the cells for 4 h. Then, the supernatant was aspirated, and the blue formazan crystals were dissolved in dimethylsulfoxide. Absorbance was measured at 570 nm in a plate

reader (Fluostar optima, BMG Labtechnologies, D-Offenburg). Seven wells per dilution were averaged, and all experiments were run in triplicate. Wells containing culture medium, but no cells were used as blank value and untreated cells were used as control representing 100% viability. The viability of the tested cells was calculated by Eq. (1).

$$Viability\ (\%) = \frac{Absorbance_{tested\ cells} - Absorbance_{blank}}{Absorbance_{control\ cells} - Absorbance_{blank}} \times 100\% \quad (1)$$

The semi-logarithmic curves were fitted using a sigmoidal function (Microcal Origin 6.0 software – OriginLab Corporation, US-Northampton). According to ISO 10993-5, a 'reduction of cell viability by more than 30% is considered a cytotoxic effect' [27]. Based on this, we used for our experiments stricter criteria and defined viability values in the range from 120% to 80% as non-toxic (no effect on cell viability). In the range between 80% and 20%, the cells were considered to have a more or less reduced viability. Values below 20% were regarded as an indication for a complete loss of viability. The IC_{50} values were determined as the concentration which reduces the cell viability to 50%. Statistical analysis was performed using Student's *t*-test.

2.2.5. Uptake studies

L929 mouse fibroblasts were plated onto glass cover slips in 24-well tissue culture plates (25,000 cells/cm^2). The cells were grown for 48 h (same conditions as described for MTT assay). Prior to incubation, the cells were washed with phosphate-buffered saline (PBS, pH 7.4). Nanoparticle dilutions (0.5% or 0.05% lipid) in culture medium were added, and the cells were incubated for 2 h at 4 °C and 37 °C or for 24 h at 37 °C. Reference cells were either non-treated or they were incubated in the presence of DiI (addition of an ethanolic DiI stock solution, resulting DiI concentration corresponded to that of a 0.5% LNP dilution). After incubation, the supernatant was aspirated, the cells were washed two times with PBS and a formaldehyde solution (3.7% in PBS, pH 7.4) was added. Incubation for 10 min in a refrigerator led to cell fixation. Subsequently after extensive washing, the cell nuclei were stained by addition of a bisbenzimide solution (5 μg/ml in PBS, pH 7.4). The cells were incubated for 30 min at room temperature and finally washed four times. The cover slips were carefully removed from the wells and mounted onto object slides in an embedding medium (14% PVA, 24% glycerol, 62% PBS). Preparations were microscopically investigated with a confocal laser scanning microscope (LSM 510 META, Carl Zeiss Jena, D-Jena). A Plan-Neofluar 40×/1.3 oil immersion objective was used. Bisbenzimide fluorescence was excited by a UV laser (351 nm) and detected after passing a 385–470 nm bandpass filter. DiI fluorescence was excited by a HeNe laser (543 nm) and detected after passing a 560–615 nm bandpass filter. Laser intensities and detector gains were usually kept similar. Exceptions were the images after 24 h of incubation and images of cells being treated with a DiI solution. In both cases, the DiI detector gain had to be reduced by about 25% (24 h) or 32% (DiI). Additional images of cells treated with Tw80-stabilized formulations were obtained with maximum laser performance and DiI detector gain. The acquired images were processed by the Zeiss LSM image browser (Carl Zeiss MicroImaging, D-Jena) with the dyes being displayed in blue (bisbenzimide) or red (DiI).

3. Results and discussion

3.1. Particle characterization

An overview of all formulations with their respective preparation conditions and PCS measurement results is provided in Table 1. The PCS *z*-average particle sizes of the LNPs were between 80 and 150 nm with corresponding PIs between 0.08 and 0.20. Only the PIs of the PVA-stabilized particles were below 0.1. The presented data were obtained from the diluted particle formulations 1–2 days prior to their application on the cells. There were no significant differences to the PCS data obtained from the undiluted formulations during the week after preparation (data not shown). The diluted reference Lipofundin® MCT (4% lipid) had a larger particle size than the LNPs but a comparable PI. The reference liposomes had a comparable particle size, but a lower PI value due to the extrusion process.

The physical state of the particle matrix was confirmed by DSC measurements (Table 1). Since the formulations contained relatively high amounts of stabilizers that, in their free, unbound form can exhibit strong cytotoxicity, measures were taken to reduce and standardize the amount of free surfactant [17,28,29]. For unbound F68, Tw80 and PVA molecules being present in the form of monomers or micelles, respectively, simple purification methods like dialysis or dilution were used. Concentrations corresponding to 2.2–2.7% unbound PVA and to 1.5–2.0% unbound Tw80 in the undiluted formulations were found. With regard to future investigations, it would be desirable to further optimize most of the methods used to standardize the concentration of unbound surfactant. For instance, it is a relatively complex procedure to dialyse the investigated LNPs without the risk of lipid recrystallization or microbial contamination. The determination of unbound Tw80 yielded fluctuating values, indicating dynamic processes, e.g. surfactant desorption during dilution. In S100/SGC formulations, the unbound stabilizer molecules form a heterogenic system of vesicles and micelles [10,30,31]. Since no appropriate method for the quantification of these compounds was found the adjustment to a specific content was not possible.

Beside the presence of unbound stabilizers in the dispersions also the particle stability in the respective incubation media is an important factor. Alterations in particle size would indicate instabilities or agglomeration [32,33]. All reference samples and formulations stabilized with the non-ionic stabilizers F68, Tw80 or PVA were stable in cell culture medium at 37 °C over the investigated incubation time (Fig. 1). The PIs were unchanged or, at maximum, increased slightly (e.g. for TM-F68 L or TM-Tw80 L and C). Also the DiI-labelled formulations did not show alterations of size or PI (data not shown). Interestingly, the charged S100/SGC particles with a crystalline matrix showed a tendency for instability (stronger increase in the PI) whereas the liquid or liquid crystalline particles were stable. Already at a low microscopic magnification (100-fold, usually used to investigate cell morphology) larger particle agglomerates were observable in the dilutions of S100/SGC-stabilized crystalline particles. Such instabilities of these particles have been observed before and they were mainly attributed to interactions with charged species in the cell culture media [34]. Especially for the crystalline S100/SGC formulations this might lead to agglomeration, since during crystallization and formation of platelet-like suspension particles, the particle surface area increases immensely. The new surface has to be covered by surfactant molecules from the aqueous phase (mixed micelles of S100/SGC or S100 vesicles) [31]. This might lead to a less effective stabilization of the formulation, which is stable enough for storage or dilution in water (e.g. for PCS measurements), but which is unstable when diluted in the charge-containing cell culture medium. In this context, a strong interaction between discrete particles could take place via their large surface areas and their prolonged edges.

As expected, cryo-TEM investigations revealed the presence of differently shaped particles in the formulations (Fig. 2). The liquid TM particles (left column) had a typical droplet shape, irrespective of the type of stabilizer. Their crystalline counterparts (second column) displayed platelet-like anisometric structures with more or less angular edges. The edges of Tw80- and S100/SGC-stabilized

Table 1
Overview on preparation and storage conditions, physical state of the matrix lipid, PCS particle sizes (*z*-average diameter) and polydispersity indices (PI) of the particle formulations under investigation.

Formulation[a]	Homogenization conditions	Storage temperature (°C)	Physical state of the matrix lipid[b]	*z*-Average (nm)[c]/PI[d]
TM-F68 L	70 °C, 3 min, 700 bar	23	Supercooled liquid	129/0.11
TM-F68-DiI[e] L	70 °C, 3 min, 700 bar	23	Supercooled liquid	122/0.12
TM-F68 C	70 °C, 3 min, 700 bar	4	Crystalline	138/0.15
TM-F68-DiI[e] C	70 °C, 3 min, 700 bar	4	Crystalline	131/0.16
CM-F68 LC	90 °C, 5 min, 850 bar	23	Supercooled smectic	142/0.12
CM-F68 C	90 °C, 5 min, 850 bar	4	Crystalline	152/0.11
TM-Tw80 L	70 °C, 3 min, 700 bar	23	Supercooled liquid	83/0.19
TM-Tw80-DiI[e] L	70 °C, 3 min, 700 bar	23	Supercooled liquid	80/0.18
TM-Tw80 C	70 °C, 3 min, 700 bar	4	Crystalline	88/0.20
TM-Tw80-DiI[e] C	70 °C, 3 min, 700 bar	4	Crystalline	87/0.20
CM-Tw80 LC	90 °C, 5 min, 850 bar	23	Supercooled smectic	125/0.12
CM-Tw80 C	90 °C, 5 min, 850 bar	4[f]	Crystalline	136/0.12
TM-S100/SGC L	70 °C, 3 min, 450 bar	23	Supercooled liquid	92/0.15
TM-S100/SGC C	70 °C, 3 min, 450 bar	4	Crystalline	102/0.16
CM-S100/SGC LC	90 °C, 5 min, 850 bar	23	Supercooled smectic	126/0.13
CM-S100/SGC C	90 °C, 5 min, 850 bar	4	Crystalline	130/0.16
TS-PVA Cα	80 °C, 3 min, 750 bar	4	crystalline (α modif.)	139/0.08
TS-PVA Cβ	80 °C, 3 min, 750 bar	4	Crystalline (β modif.)	145/0.09
Lipofundin® MCT	–	23	Liquid	256/0.14
Liposomes	–	4	–	112/0.07

[a] The letters L, LC, C indicate the physical state of the LNPs.
[b] Confirmed by DSC measurements.
[c] All standard deviations of the *z*-average were below 1 nm (0.1 nm to 0.7 nm).
[d] All standard deviations of the PI were below 0.01 (0.001–0.007).
[e] Particles labelled with DiI (1 mg/g lipid).
[f] Seven days of storage at 0 °C prior to storage at 4 °C.

platelets appeared to be smoother than those of the polygonal F68-stabilized platelets. The Tw80- and S100/SGC-stabilized supercooled liquid crystalline CM particles had a clearly cylindrical, barrel-like shape (third column) whereas the respective F68-stabilized 'barrels' appeared to be rather swollen [9,35,36]. Crystalline CM particles (right column) showed a regularly formed platelet-like shape. Their edges were in all cases very smooth and rounded, and thus, the platelets appeared almost round or oblong. The micrographs also revealed the presence of additional colloidal structures in the dispersions (e.g. micelles or ring-shaped liposomes in S100/SGC-stabilized formulations) [10,30,31]. As described previously [13], the crystalline Cα and Cβ TS-PVA particles had a significantly different particle shape (Fig. 3). The circular shape of Cα particles was comparable with that of TM emulsion droplets. The Cβ particles were rather angularly shaped platelets. Incubation of the Cα sample for 2 h at 37 °C prior to cryo-preparation led to the appearance of angular edges at the still rounded Cα particles. In agreement with the DSC and X-ray measurements (see Supporting Information), a structural transition seemed to have started.

3.2. Cell viability – reference samples

The parenteral fat emulsion Lipofundin® MCT and soybean phospholipid liposomes were chosen as reference samples because the physiological tolerability of such formulations is well established as reflected in their extensive clinical use [37,38]. Their high cell compatibility was also obvious from the high viability values observed over the whole range of investigated lipid concentrations in the present study (Fig. 4A and B). The extension of the incubation period to 24 h led to only a slight reduction in the cell viability but the viability never dropped below 50%. The determination of an IC_{50} value was thus not possible for these reference formulations under the chosen conditions. It can be assumed to be larger than 1% lipid, since this was the highest lipid concentration under investigation (see below). For the parenteral fat emulsion Lipofundin® MCT, this concentration was much higher than the usual clinical doses. A bolus injection (0.1 g lipid/kg body weight) to a person of 70 kg body weight and 6 L of blood would cause a maximal blood concentration slightly above 0.1% lipid (m/m), which would be eliminated within minutes [39]. However, parenteral fat emul-

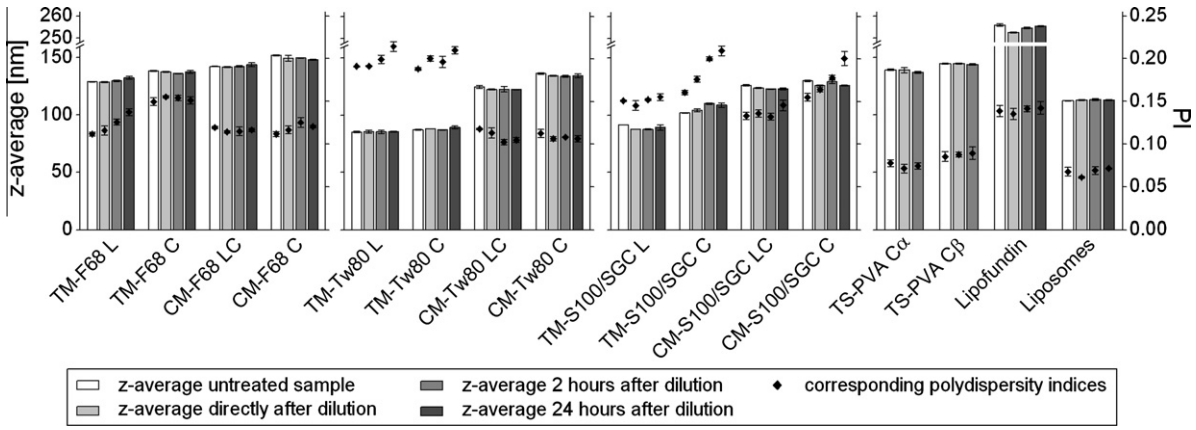

Fig. 1. Stability of the particle formulations in cell culture medium (RPMI 1640 supplemented with 10% foetal calf serum and 4 mM L-glutamine).

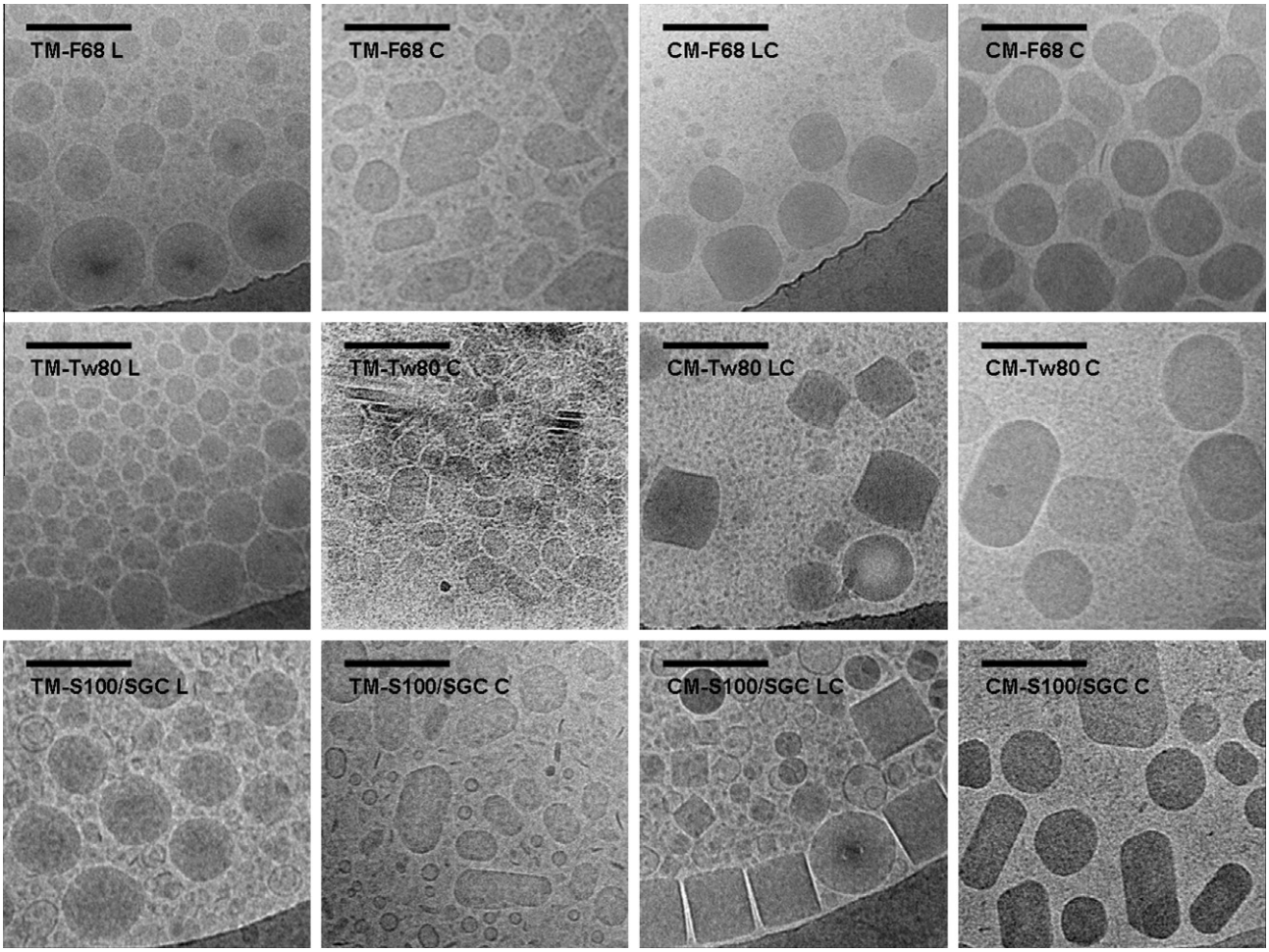

Fig. 2. Cryo-electron micrographs of the particle formulations. Left column – supercooled liquid (L) TM formulations; second column – crystalline (C) TM formulations; third column – supercooled smectic (LC) CM formulations; right column – crystalline (C) CM formulations; the scale bars represent 200 nm. Please note that the three-dimensional shape of the particles is displayed as a two-dimensional projection in the electron micrographs. Platelets are most often displayed as elongated or round structures in top-view and cylindrical particles may appear either as rectangular (side-view) or spherical (top-view).

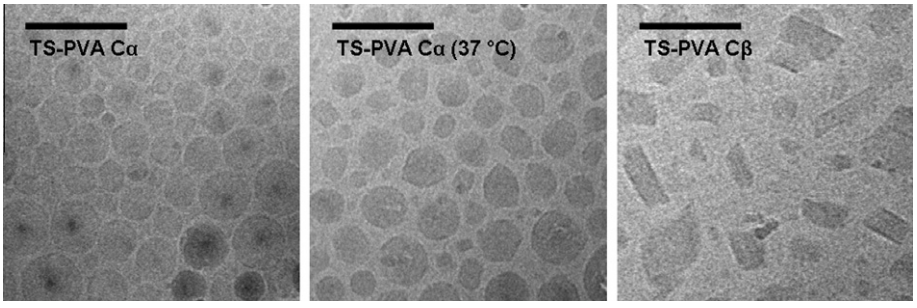

Fig. 3. Cryo-electron micrographs of the crystalline TS-PVA particle formulations. Left – α-modification of TS (Cα); middle – Cα sample pretreated by 2 h incubation at 37 °C; right – β-modification of TS (Cβ); the scale bars represent 200 nm.

sions are usually not administered as bolus, and thus, during infusion of this dose per hour (clinically relevant dosing), the lipid concentration would be at a plateau of about 0.06% [39]. Also, bolus injections of drug-loaded fat emulsions like Propofol-®Lipuro would result in much lower blood lipid concentrations (0.01–0.03%).

In order to confirm the response of the test, thiomersal, a preservative used in vaccines or in LNP formulations, was chosen as a reference causing cell death [40]. Usually, after incubation with 0.02% thiomersal, the viability was around 1–2% (data not shown).

All LNP test formulations applied in this study were standardized to a lipid matrix content of 4% in an isotonic surfactant- and glycerol-containing aqueous phase. To obtain the respective concentration series applied to the cells, these stock formulations were diluted with cell culture medium. If very high lipid concentrations were used, only a small volume of cell culture medium could be added to the stock formulations (e.g. a 1:1 dilution would be required to achieve 2% lipid concentration). In such a mixture, the amount of nutritious and buffering culture medium ingredients is quite low, which might affect the viability of the cells. In order

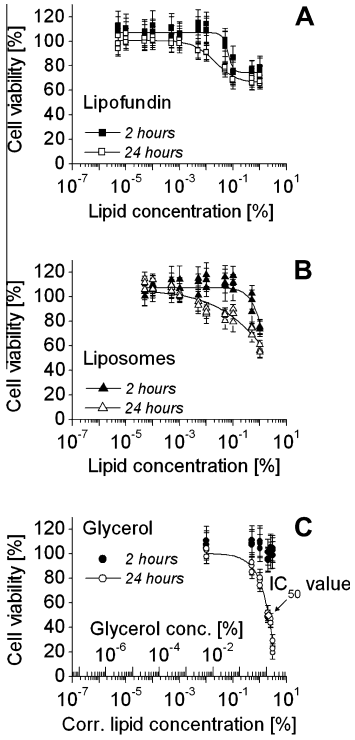

Fig. 4. Viability of L929 mouse fibroblasts after incubation with Lipofundin® MCT (A), liposomes (B) and with a glycerol solution (C) for 2 or 24 h. The second scaling of the abscissa in C ('Corr. lipid concentration') relates the results to that of formulations composed of 4% lipid in a stabilizer containing glycerol solution (2.25%).

to separate a potentially resulting starving effect on the cells from that of the lipid particles, dilutions of a pure isotonic glycerol solution with cell culture medium were tested with regard to their effect on cell viability (Fig. 4C). All tested dilutions had a physiological osmolarity. The most 'concentrated' glycerol dilution corresponded to a lipid concentration of 3% (if a particle formulation would have been applied) and was tolerated well during an incubation time of 2 h. However, after an incubation time of 24 h, the cell viability had decreased, probably as a result of a decreased pH due to a lack of buffering agents and as a result of insufficient supply of nutritious ingredients. Since after 24 h about 80–90% viability was still obtained from dilutions corresponding to 0.5–1% lipid in the mixture, all investigations on particle formulations or stabilizer solutions were performed with dilutions not exceeding these values.

3.3. Cell viability – LNP formulations

The viability curves of mouse fibroblasts treated with the differently stabilized LNP formulations and the corresponding stabilizer solutions/dispersions for 2 or 24 h are displayed in Fig. 5. The results were highly reproducible and, with only a few exceptions, the semi-logarithmic relation between formulation concentration and cell viability had the expected sigmoidal progression. However, viabilities at the respective lipid concentrations were in all cases lower than compared with the reference samples. This was also reflected by the IC_{50} values determined from the fitted curves (Fig. 6). Usually, a longer incubation period (24 h) led to lower IC_{50} values than a short incubation period (2 h). Only some of the stabilizer formulations showed a high tolerability, and thus, the IC_{50} values could not be determined (S100/SGC dispersion after 2 h incubation, F68 solution, PVA solution).

3.3.1. Effect of physical state

The most striking result from the cell viability investigations was the consistently lower cell compatibility of crystalline versus the corresponding liquid or liquid crystalline LNPs (Figs. 5 and 6). This was reflected by the low viability and the low IC_{50} values of cells treated with the crystalline particles. The physical state of the matrix lipid thus had a pronounced effect on the *in vitro* cell viability. Although the IC_{50} values of the different LNP formulations were distributed over a range of lipid concentrations of about three orders of magnitude (Fig. 6), it became obvious that the tolerability by the cells increased from crystalline over supercooled liquid crystalline to supercooled liquid lipid matrices. Furthermore, the differences between liquid and crystalline TM matrices (Fig. 5H–M) were more pronounced than those caused by liquid crystalline and crystalline CM matrices (Fig. 5O–T). The comparatively good tolerability of a liquid matrix is in agreement with the high viabilities after incubation with Lipofundin® MCT.

3.3.2. Effect of particle shape

A reduction in cell viability and IC_{50} values in the order spherical (liquid TM) to barrel-shaped (liquid crystalline CM) to platelet-like particles (crystalline TM and CM) was detected. Thus, the less spherical and the more angular the particle shape, the lower the cell viability and the IC_{50} values. However, it has to be pointed out that the parameter 'particle shape' is closely related to the parameter 'physical state' of the matrix lipid and the results regarding particle shape correlate with the respective results regarding physical state. Thus, the observations of the present study might result from either the effect of particle shape or physical state or from an overlap of the two parameters. In an attempt to distinguish between these effects, crystalline TS-PVA particles were prepared in two different polymorphic forms. In the α-modification, these particles are spherical but they assume an angular, platelet-like shape in the β-modification [13]. However, the differences in shape between the TS particles in the two crystal modifications were not as pronounced as observed, e.g. for liquid and crystalline TM LNPs. Moreover, according to the results of physicochemical characterization, e.g. electron microscopic investigations (Fig. 3), slight modifications of the particle properties occurred during the 2 h of incubation with the cells. In any case, after 2 h of incubation, these differently shaped crystalline TS particles showed very similar viability curves and IC_{50} values (Fig. 5N). However, different viability curves and IC_{50} values would have been expected if the particle shape would have played a major role regarding the effect on viability. Thus, these findings can be taken as a first indication that rather the physical state of the matrix lipid than the particle shape led to the observed effects on the cell viability.

3.3.3. Effect of the matrix lipid

It is also interesting to compare the impact of the formulation composition (lipid, stabilizer) on cell viability and IC_{50} values. Conclusions regarding the influence of the type of lipid matrix (TM versus CM) could only be drawn from the results of the crystalline LNPs in which the physical state of the lipids is reasonably comparable (in contrast to that of liquid TM droplets and liquid crystalline CM 'barrels').

The results may point to a slightly higher tolerability of the lipid CM as reflected in higher IC_{50} values of the F68- and Tw80-stabilized CM LNPs (Fig. 6). The effects may, however, be superimposed by those of other parameters like differences in particle size (in particular for the Tw80 LNPs) and particle shape (particularly for the F68 LNPs). Nevertheless, the findings regarding the effect of particle shape (differently shaped crystalline TS-PVA LNPs) would support the assumption that rather the lipid than the particle shape caused the differences.

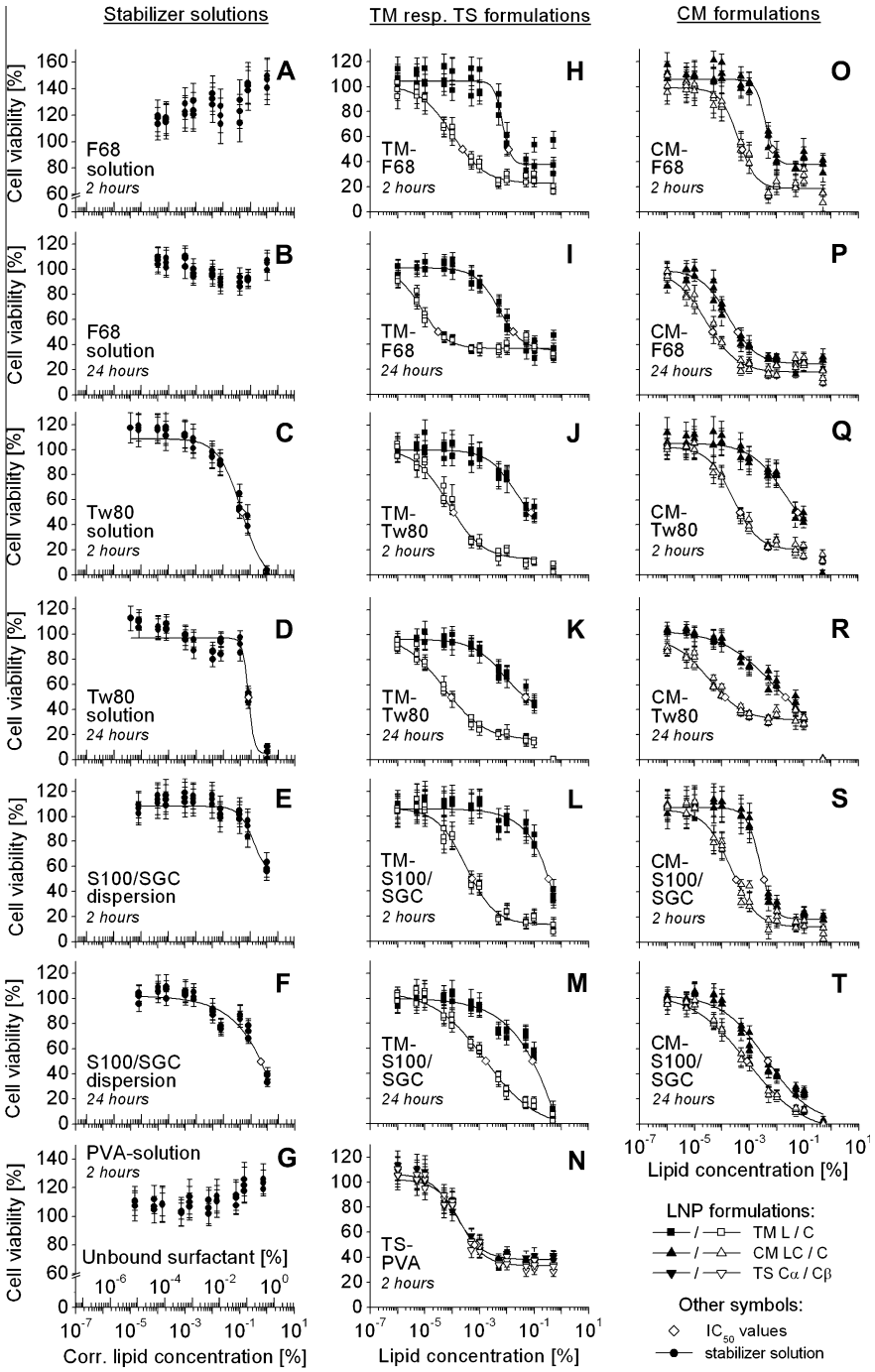

Fig. 5. Viability of L929 mouse fibroblasts after 2 or 24 h of incubation with stabilizer solutions (left column), TM LNPs (middle column, lines 1–6), TS LNPs (middle column, line 7) and CM LNPs (right column) stabilized with F68 (lines 1–2), Tw80 (lines 3–4), S100/SGC (line 5–6) or PVA (line 7). The second scaling of the abscissa of the viability curves after incubation with stabilizer solutions is based on the formulation composition of 4% lipid and 1.6% unbound stabilizer in the aqueous phase (was not determined for S100/SGC, 2.2% for PVA) and on the assumption that the stabilizer is not desorbed from the particles during dilution.

In comparison with Tw80 and F68, for the crystalline S100/SGC LNPs, slightly higher IC_{50} values were determined for TM. However, when interpreting the results of the S100/SGC-stabilized platelets, their above-mentioned instabilities have to be taken into consideration. Usually, an increase in incubation time led to decreasing or almost consistent IC_{50} values. But for the crystalline S100/SGC LNPs, the viability curves proceeded almost linearly and the IC_{50} values had increased in a statistically significant manner after 24 h of incubation (Figs. 5M and T and 6). Thus, it is not clear whether TM really showed a higher tolerability in this case, or whether the differences were solely caused by the instabilities.

Regarding the effect of the matrix lipid on the cell viability it would be interesting to extend the investigations, e.g. by evaluating the effects of series of different triglycerides or cholesterol esters in future studies. In preliminary investigations, the replacement of the triglyceride TM by trilaurin (TL, see Supporting Information) did not yield significant differences between the tested supercooled liquid LNPs from the two triglycerides

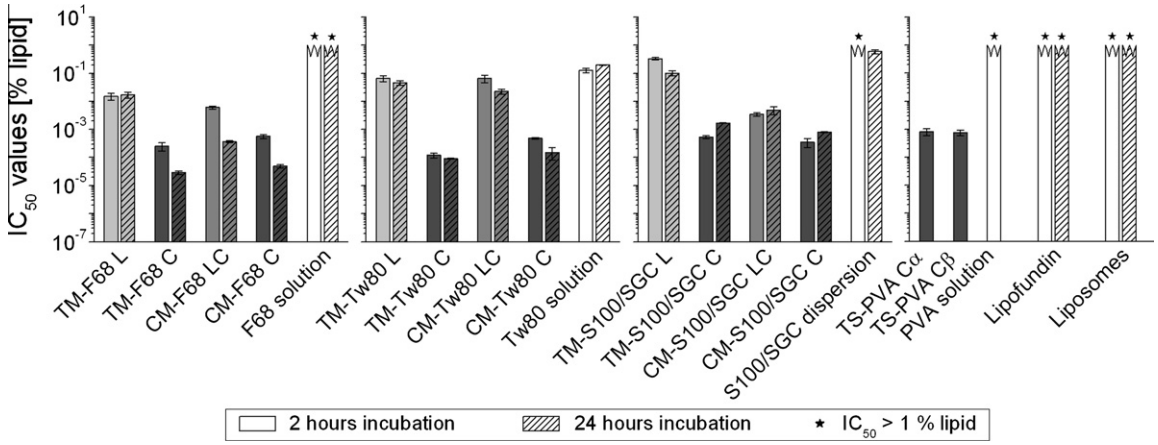

Fig. 6. IC_{50} values of the tested formulations after 2 or 24 h incubation. White bars – reference solutions/formulations, light grey – supercooled liquid matrix (L), grey – supercooled smectic matrix (LC), dark grey – crystalline matrix (C). Formulations, where the IC_{50} could not be determined in the investigated range of lipid concentrations are displayed as columns ending with a jagged line.

stabilized with Tw80. These results indicate that slight changes of the matrix lipid (i.e. length of the carbon chain) exert no or only marginal effects on the cell viability.

3.3.4. Effect of the stabilizer

In order to prepare stable LNP formulations, at least one stabilizing surfactant is needed. Surfactants, especially the fraction which is not bound to the particle surface but present in the aqueous phase, can lead to adverse effects on the cell viability [17,19,28,29]. However, F68 is a surfactant with only negligible lytic activity [41]. Treating the cells with a pure F68 solution (Fig. 5A and B) led to strongly increased (~150%, 2 h) or very high viability values (~110%, 24 h). This might be a result of the cell protective effect of F68 [42]. The stabilizer is able to interact with cell membranes, thereby sealing membrane damages, preventing apoptosis and supporting cell recovery [43–45]. Such a cell protective effect with increased cell viabilities was also induced by a PVA solution (Fig. 5G) [42,46]. In contrast, high concentrations of a pure Tw80 solution caused a strong reduction in the cell viability (Fig. 5C and D). At Tw80 concentrations corresponding to 0.5% or 1% matrix lipid (if a LNP formulation would have been applied), the cells were rounded and partially detached from the well bottom. Furthermore, microscopically visible black spots distributed all over the cell surface indicated a membrane damaging effect of Tw80 [47]. In a Tw80-stabilized LNP formulation, this toxic effect of unbound Tw80 superimposes the effect of the LNPs. This conclusion is supported by the extraordinarily strong decrease in the cell viability at the highest lipid concentration (0.5%), especially after 24 h of incubation with Tw80-stabilized LNP formulations. This lipid concentration was, therefore, excluded from the sigmoidal fit of the viability values for the Tw80 LNPs. A reduction in the amount of unbound Tw80 (and also of the other stabilizers) was not possible since particle instabilities were expected. A pure S100/SGC dispersion led to viability curves between those obtained for F68 and Tw80 solutions (Fig. 5E and F). The curves were highly similar to those obtained for the reference S100 liposomes. However, the scaling of Fig. 5E and F is not exact with regard to the amount of unbound S100/SGC since the unbound complex mixture could not be determined quantitatively in the LNP formulations. Thus, 1.6% S100/SGC was assumed to be unbound as this was also the adjusted value for the F68 and Tw80 formulations.

According to the results obtained with the pure stabilizers, the LNP formulations were expected to reduce the cell viability in the order F68 to S100/SGC to Tw80-stabilized particles. Surprisingly, the F68-stabilized LNPs caused the lowest IC_{50} values of all investigated formulations, whereas the relatively toxic stabilizer Tw80, bound to the LNPs, led to quite high IC_{50} values. Only after 2 h incubation, the crystalline Tw80 platelets led to slightly lower IC_{50} values than the F68 platelets which would be consistent with literature reports on crystalline TM platelets [16]. The liquid TM-S100/SGC LNPs led to the highest IC_{50} values and thus had the best cell compatibility. This is not unexpected since the composition of this formulation is similar to that of the phospholipid-stabilized emulsion droplets used as biocompatible reference sample. The slightly higher toxicity of TM-S100/SGC L might be related to the presence of the bile salt SGC [48]. A general conclusion regarding the effect of the single stabilizers in the LNP formulation could, however, not be drawn except that S100/SGC-and Tw80-stabilized LNPs were better tolerated than F68-stabilized formulations.

3.4. Cellular uptake of TM LNPs

Several mechanisms are able to induce cytotoxicity of LNPs. One key factor might be a high particle uptake [15]. In order to evaluate this potential mechanism, cell uptake studies were conducted using DiI-labelled LNPs with a TM matrix. TM particles were chosen because the differences regarding the effect on cell viability were more pronounced between the liquid and the crystalline TM particles than between the liquid crystalline and the crystalline CM particles. Confocal micrographs did indeed point to a relatively high uptake of all DiI-labelled TM-F68 formulations (Fig. 7) within a short time whereas the uptake of TM-Tw80 formulations (Fig. 8) was almost negligible. For comparison, cells were treated with a DiI solution (Fig. 7) leading to a strong red fluorescence, which, especially after 24 h, was partially localized in granular structures surrounding the nucleus. Since the fluorescence intensity was much stronger than from the LNP formulations, the images had to be obtained with a reduced detector gain and a direct comparison, e.g. to the F68 LNPs, is thus not possible.

Incubated at 37 °C, the F68 LNPs showed a strong red fluorescence which was mostly located in spherical compartments within the cell, as confirmed by optical slices along the *z*-axis (Fig. 7). According to the time-dependent distribution pattern, the LNPs seem to have entered the cells via an endocytotic pathway and were present in endosomal/lysosomal compartments. In addition, adsorptive mechanisms (e.g. via an interaction of F68 and cell membrane) might have influenced the toxic effect of the LNPs. This way of interaction with the cells was recognizable by the strong fluorescence from the cell surface, which was detectable after 2 h incubation with high F68 LNP concentrations (0.5% lipid) at 37 °C

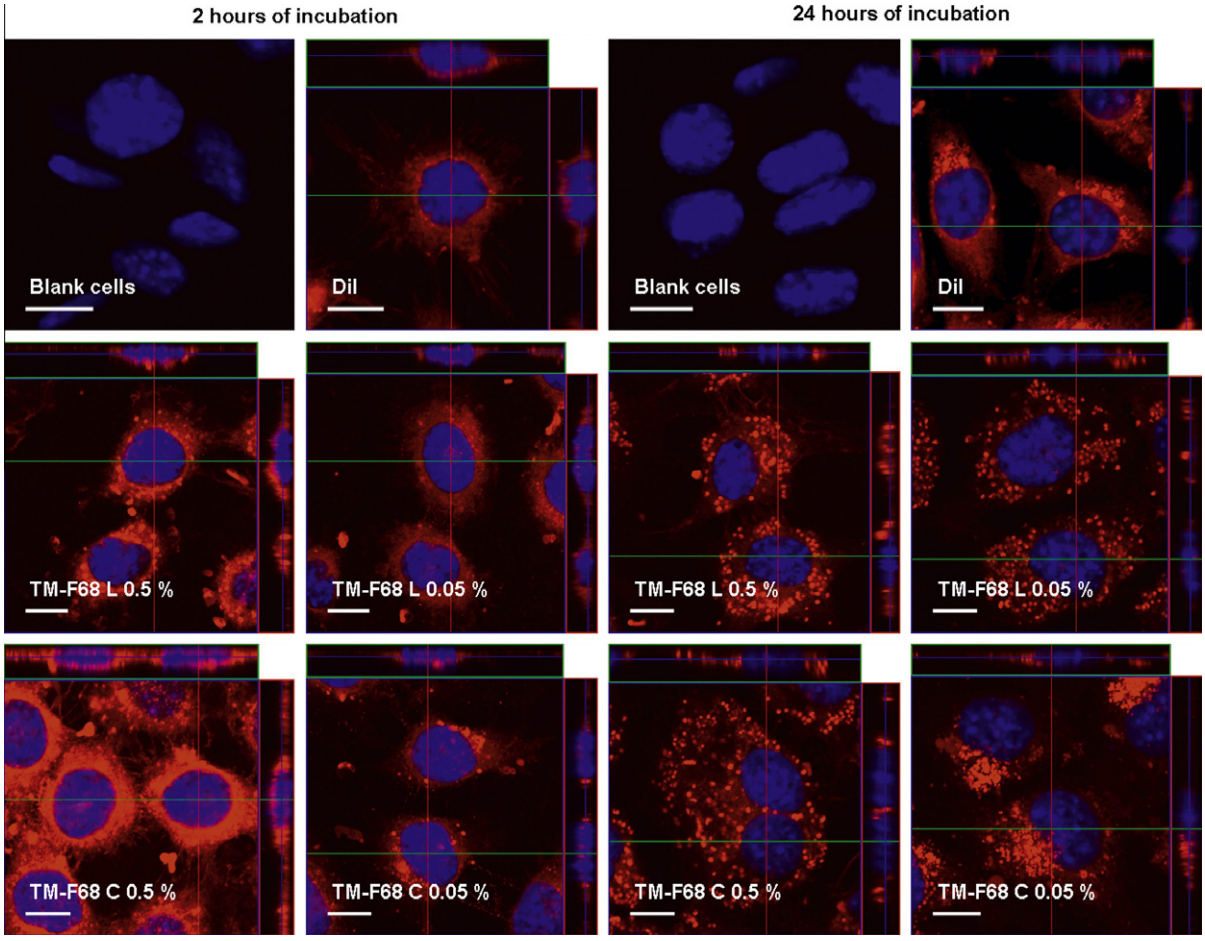

Fig. 7. Confocal laser scanning micrographs of L929 mouse fibroblasts after 2 or 24 h incubation at 37 °C (the scale bars represent 10 μm, the cell nuclei were stained with bisbenzimide (blue), the LNPs were labelled with DiI (red)). First line – untreated blank cells and cells treated with DiI stock solution; second line – cells treated with supercooled liquid (L) TM-F68 LNPs; third line – cells treated with crystalline (C) TM-F68 LNPs. Usually, the micrographs were obtained under the same conditions (exceptions: 24 h of incubation and treatment with DiI required a reduction in DiI detector gain (see text)).

but also at 4 °C (micrographs not shown). Where distinguishable, the fluorescence of cells treated with crystalline, platelet-like particles seemed to be stronger than that of cells treated with liquid emulsion droplets. Furthermore, the uptake of the LNPs in a concentration of 0.5% lipid appeared to be stronger than that of 0.05% lipid.

Investigated under the same conditions, cells treated with TM-Tw80 LNPs exhibited almost no fluorescence, although containing the same amount of DiI as the F68 LNPs (Fig. 8, first row). Only a minor amount of particles had been taken up by the cells which only became visible with increased laser power and DiI detector gain (Fig. 8, second row). After 2 h, no matter if incubated at 37 °C or at 4 °C, the fluorescence intensity was slightly stronger than from untreated cells visualized under the same conditions (micrographs not shown). Only after 24 h, globular diffuse fluorescent compartments were observable within the cell cytoplasm. Their size appeared to be larger than the size of the circular structures in the TM-F68-treated cells. Probably, fusion processes influenced by Tw80 might have led to these rather extended compartments. Incubation with a lipid concentration of 0.5% led to cell lysis caused by unbound Tw80 and thus no cells could be recovered for microscopical imaging.

The results obtained by confocal microscopy could be confirmed and quantified by flow cytometric investigations (see Supporting Information). However, flow cytometry revealed that the difference in fluorescence intensity between liquid and crystalline LNPs was only significant for Tw80 LNPs but not for F68 LNPs. Nevertheless, in correlation to the viability data, for both stabilizers, the uptake of the crystalline LNPs was higher than that of the liquid LNPs. This stronger uptake of crystalline LNPs might have led to the decreased cell viabilities measured in the MTT assay. But since the observed differences in particle uptake were of minor extent compared with the strong differences in viability, additional influencing factors must be assumed. On the other hand, the detection of only small differences might also be related to the application of relatively high LNP concentrations for the uptake studies. These high concentrations had to be applied in order to microscopically visualize any uptake (a slightly reduced lipid concentration was applied during flow cytometric investigation, see Supporting Information). For F68 LNPs, the correlating viabilities were low and in a range with only minor differences between liquid and crystalline particles (i.e. in the right part of the curves of Fig. 5H and I). For Tw80, one of the applied concentrations led to cell lysis. The second concentration was in a range where the emulsion droplets caused a significantly higher viability than the crystalline platelets. But since the uptake of the particles was very low, differences were hardly visible. However, the crystalline particles resulted in a higher uptake, which would be explainable by the particle shape. A flat platelet attached to the cell surface has a larger contact area than a sphere and is much less exposed to shear stress arising, e.g. from rinsing the cells. Platelets could thus adhere much better probably leading to a better uptake [49]. After being endocytosed, it seems

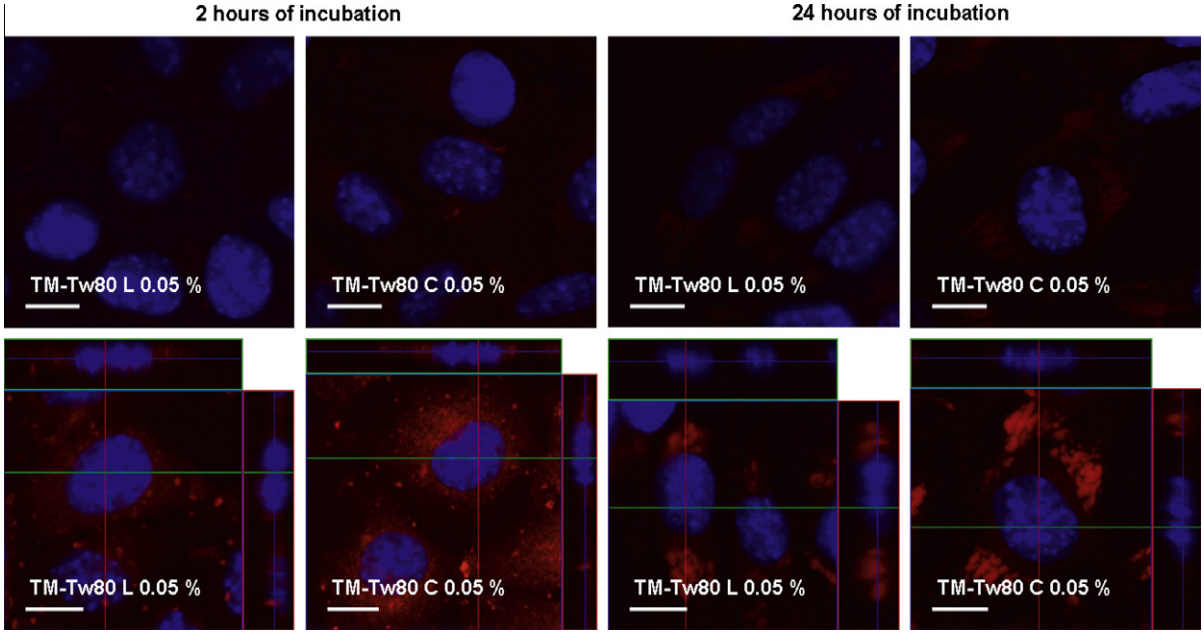

Fig. 8. Confocal laser scanning micrographs of L929 mouse fibroblasts after 2 or 24 h incubation with TM-Tw80 LNPs in the supercooled liquid (L) and in the crystalline (C) state (the scale bars represent 10 μm, the cell nuclei were stained with bisbenzimide (blue), the LNPs were labelled with DiI (red)). Top line – the micrographs were obtained under the same conditions as for TM-F68 formulations (Fig. 7); bottom line – the micrographs were obtained with maximum laser performance and DiI detector gain.

conceivable that the trafficking of the particles and their metabolism by the cells is different, e.g. for crystalline platelets or spherical droplets, but such factors need to be further investigated before a final explanation can be given.

4. Conclusions

All investigated LNP formulations had a more or less pronounced effect on the *in vitro* cell viability of L929 mouse fibroblasts. The most influencing factor was found to be the physical state of the matrix lipid. Supercooled liquid LNPs caused the least reduction in cell viability. The IC_{50} values of the corresponding crystalline LNPs were up to three orders of magnitude lower and thus, these particles led to the strongest reduction in cell viability. The supercooled smectic CM LNPs were comparably or less tolerable than the respective TM emulsion droplets. Also for CM LNPs, a decrease in cell viability was observed after crystallization. The particle shape is closely related to the physical state of the matrix lipid and an influence of the particle shape on the cell viability might be suggested. However, the influence of the shape on the cell viability seemed to be of minor extent. Nevertheless, this aspect of our study calls for further investigations. There were also some correlations between cell viability and formulation composition. The cholesterol ester CM was slightly better tolerated by the cells than the triglyceride TM. Regarding the stabilizing surfactants different effects were found for stabilizer solutions/dispersions and the respective LNP formulations. The most outstanding finding was the strong reduction in cell viability by LNPs stabilized with F68. This was mainly attributed to a strong cellular uptake of these particles, but also other factors (e.g. particle adsorption) might play a role. The mechanisms of particle interaction with the cell (uptake, adsorption) and the fate of the particles within the cell will have to be elucidated in further studies.

Acknowledgement

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejpb.2011.03.022.

References

[1] S.M. Moghimi, A.C. Hunter, J.C. Murray, Nanomedicine: current status and future prospects, FASEB J. 19 (2005) 311–330.
[2] P.P. Constantinides, M.V. Chaubal, R. Shorr, Advances in lipid nanodispersions for parenteral drug delivery and targeting, Adv. Drug Deliv. Rev. 60 (2008) 757–767.
[3] P. Couvreur, C. Vauthier, Nanotechnology: intelligent design to treat complex disease, Pharm. Res. 23 (2006) 1417–1450.
[4] W. Mehnert, K. Mäder, Solid lipid nanoparticles. Production, characterization and applications, Adv. Drug Deliv. Rev. 47 (2001) 165–196.
[5] H. Bunjes, B. Siekmann, Manufacture, characterization, and applications of solid lipid nanoparticles as drug delivery systems, in: S. Benita (Ed.), Drugs and the Pharmaceutical Sciences, vol. 158, CRC Press, Boca Raton, 2006, pp. 213–268 (Microencapsulation – Methods and Industrial Applications, second ed.).
[6] K. Westesen, H. Bunjes, Do nanoparticles prepared from lipids solid at room temperature always possess a solid lipid matrix?, Int J. Pharm. 115 (1995) 129–131.
[7] H. Bunjes, K. Westesen, M.H.J. Koch, Crystallization tendency and polymorphic transitions in triglyceride nanoparticles, Int. J. Pharm. 129 (1996) 159–173.
[8] K. Westesen, H. Bunjes, M.H.J. Koch, Physicochemical characterization of lipid nanoparticles and evaluation of their drug loading capacity and sustained release potential, J. Controlled Release 48 (1997) 223–236.
[9] J. Kuntsche, K. Westesen, M. Drechsler, M.H.J. Koch, H. Bunjes, Supercooled smectic nanoparticles: a potential novel carrier system for poorly water soluble drugs, Pharm. Res. 21 (2004) 1834–1843.
[10] K. Westesen, Novel lipid-based colloidal dispersions as potential drug administration systems – expectations and reality, Colloid Polym. Sci. 278 (2000) 608–618.
[11] K. Westesen, M. Drechsler, H. Bunjes, Colloidal dispersions based on solid lipids, in: E. Dickinson, R. Miller (Eds.), Food Colloids: Fundamentals of Formulation, Royal Society of Chemistry, Cambridge, 2001, pp. 103–115.
[12] H. Bunjes, F. Steiniger, W. Richter, Visualizing the structure of triglyceride nanoparticles in different crystal modifications, Langmuir 23 (2007) 4005–4011.
[13] K.M. Rosenblatt, H. Bunjes, Poly(vinyl alcohol) as emulsifier stabilizes solid triglyceride drug carrier nanoparticles in the α-modification, Mol. Pharmaceutics 6 (2008) 105–120.

[14] J. Kuntsche, M.H.J. Koch, A. Fahr, H. Bunjes, Supercooled smectic nanoparticles: influence of the matrix composition and in vitro cytotoxicity, Eur. J. Pharm. Sci. 38 (2009) 238–248.
[15] R.H. Müller, S. Maaßen, H. Weyhers, F. Specht, J.S. Lucks, Cytotoxicity of magnetite-loaded polylactide, polylactide/glycolide particles and solid lipid nanoparticles, Int. J. Pharm. 138 (1996) 85–94.
[16] N. Schöler, C. Olbrich, K. Tabatt, R.H. Müller, H. Hahn, O. Liesenfeld, Surfactant, but not the size of solid lipid nanoparticles (SLN) influences viability and cytokine production of macrophages, Int. J. Pharm. 221 (2001) 57–67.
[17] R.H. Müller, D. Rühl, S. Runge, K. Schulze-Forster, W. Mehnert, Cytotoxicity of solid lipid nanoparticles as a function of the lipid matrix and the surfactant, Pharm. Res. 14 (1997) 458–462.
[18] N. Schöler, H. Hahn, R.H. Müller, O. Liesenfeld, Effect of lipid matrix and size of solid lipid nanoparticles (SLN) on the viability and cytokine production of macrophages, Int. J. Pharm. 231 (2002) 167–176.
[19] A.V. Heydenreich, R. Westmeier, N. Pedersen, H.S. Poulsen, H.G. Kristensen, Preparation and purification of cationic solid lipid nanospheres – effects on particle size, physical stability, and cell toxicity, Int. J. Pharm. 254 (2003) 83–87.
[20] W. Weyenberg, P. Filev, D. Van den Plas, J. Vandervoort, K. De Smet, P. Sollie, A. Ludwig, Cytotoxicity of submicron emulsions and solid lipid nanoparticles for dermal application, Int. J. Pharm. 337 (2007) 291–298.
[21] H. Weyhers, S. Ehlers, H. Hahn, E.B. Souto, R.H. Müller, Solid lipid nanoparticles (SLN) – effects of lipid composition on in vitro degradation and in vivo toxicity, Pharmazie 61 (2006) 539–544.
[22] L.S.C. Wan, P.F.S. Lee, CMC of polysorbates, J. Pharm. Sci. 63 (1974) 136–137.
[23] J.H. Finley, Spectrophotometric determination of poly(vinyl alcohol) in paper coatings, Anal. Chem. 33 (1961) 1925–1927.
[24] G. Dalwadi, H.A.E. Benson, Y. Chen, Comparison of diafiltration and tangential flow filtration for purification of nanoparticle suspensions, Pharm. Res. 22 (2005) 2152–2162.
[25] T. Mosmann, Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays, J. Immunol. Methods 65 (1983) 55–63.
[26] D. Fischer, Y. Li, B. Ahlemeyer, J. Krieglstein, T. Kissel, In vitro cytotoxicity testing of polycations: influence of polymer structure on cell viability and hemolysis, Biomaterials 24 (2003) 1121–1131.
[27] ISO 10993-5: 2009, Biological evaluation of medical devices – part 5: tests for in vitro cytotoxicity, 2009.
[28] E.H. Gokce, G. Sandri, M.C. Bonferoni, S. Rossi, F. Ferrari, T. Gueneri, C. Caramella, Cyclosporine A loaded SLNs: evaluation of cellular uptake and corneal cytotoxicity, Int. J. Pharm. 364 (2008) 76–86.
[29] J. Kristl, K. Teskac, M. Milek, I. Mlinaric-Rascan, Surface active stabilizer Tyloxapol in colloidal dispersions exerts cytostatic effects and apoptotic dismissal of cells, Toxicol. Appl. Pharmacol. 232 (2008) 218–225.
[30] H. Bunjes, Characterization of solid lipid nano- and microparticles, in: C. Nastruzzi (Ed.), CRC Press LLC, Boca Raton, 2005, pp. 41–66.
[31] K. Westesen, B. Siekmann, Investigation of the gel formation of phospholipid-stabilized solid lipid nanoparticles, Int. J. Pharm. 151 (1997) 35–45.
[32] A. Petri-Fink, B. Steitz, A. Finka, J. Salaklang, H. Hofmann, Effect of cell media on polymer coated superparamagnetic iron oxide nanoparticles (SPIONs): colloidal stability, cytotoxicity, and cellular uptake studies, Eur. J. Pharm. Biopharm. 68 (2008) 129–137.
[33] F. Liu, D. Liu, Long-circulating emulsions (oil-in-water) as carriers for lipophilic drugs, Pharm. Res. 12 (1995) 1060–1064.
[34] S. Petersen, H. Bunjes, Stability of different lipid nanoparticle formulations in various media, in: Controlled Release Society German Chapter Annual Meeting: Jena, Controlled Release Society Germany Local Chapter, 2006, Abstract P17.
[35] J. Kuntsche, M.H.J. Koch, M. Drechsler, H. Bunjes, Crystallization behavior of supercooled smectic cholesteryl myristate nanoparticles containing phospholipids as stabilizers, Colloids and Surfaces B: Biointerfaces 44 (2005) 25–35.
[36] J. Kuntsche, M.H.J. Koch, F. Steiniger, H. Bunjes, Influence of stabilizer systems on the properties and phase behavior of supercooled smectic nanoparticles, J. Colloid Interface Sci. 350 (2010) 229–239.
[37] M. Wirtitsch, B. Wessner, A. Spittler, E. Roth, T. Volk, L. Bachmann, M. Hiesmayr, Effect of different lipid emulsions on the immunological function in humans: a systematic review with meta-analysis, Clin. Nutr. 26 (2007) 302–313.
[38] B. Dupont, Overview of the lipid formulations of amphotericin B, J. Antimicrob. Chemother. 49 (2002) 31–36.
[39] U. Julius, W. Leonhardt, Elimination and metabolism of a fat emulsion containing medium chain triglycerides (Lipofundin MCT 10%), J. Parenteral Enteral Nutr. 12 (1988) 116–120.
[40] N. Schöler, E. Zimmermann, U. Katzfey, H. Hahn, R.H. Müller, O. Liesenfeld, Preserved solid lipid nanoparticles (SLN) at low concentrations do cause neither direct nor indirect cytotoxic effects in peritoneal macrophages, Int. J. Pharm. 196 (2000) 235–239.
[41] S.D. Singh-Joy, V.C. Mclain, Safety assessment of poloxamers 101, 105, 108, 122, 123, 124, 181, 182, 183, 184, 185, 188, 212, 215, 217, 231, 234, 235, 237, 238, 282, 284, 288, 331, 333, 334, 335, 338, 401, 402, 403, and 407, poloxamer 105 benzoate, and poloxamer 182 dibenzoate as used in cosmetics, Int. J. Toxicol. 27 (2008) 93–128.
[42] J.D. Michaels, E.T. Papoutsakis, Polyvinyl alcohol and polyethylene glycol as protectants against fluid-mechanical injury of freely-suspended animal cells (CRL 8018), J. Biotechnol. 19 (1991) 241–257.
[43] S.A. Maskarinec, J. Hannig, R.C. Lee, K.Y. Lee, Direct observation of poloxamer 188 insertion into lipid monolayers, Biophys. J. 82 (2002) 1453–1459.
[44] G. Serbest, J. Horwitz, M. Jost, K.A. Barbee, Mechanisms of cell death and neuroprotection by poloxamer 188 after mechanical trauma, FASEB J. 20 (2006) 308–310. 10.
[45] S.L. Frey, D. Zhang, M.A. Carignano, I. Szleifer, K.Y. Lee, Effects of block copolymer's architecture on its association with lipid membranes: experiments and simulations, J. Chem. Phys. 127 (2007). 114904-1-114904/12.
[46] Y. Shintani, K. Iwamoto, K. Kitano, Polyethylene glycols for promoting the growth of mammalian cells, Appl. Microbiol. Biotechnol. 27 (1988) 533–537.
[47] S.I. Simoes, J.M. Tapadas, C.M. Marques, M.E.M. Cruz, M.B.F. Martins, G. Cevc, Permeabilisation and solubilisation of soybean phosphatidylcholine bilayer vesicles, as membrane models, by polysorbate, Tween 80, Eur. J. Pharm. Sci. 26 (2005) 307–317.
[48] L. Joergensen, P. Artursson, E. Bechgaard, Toxicological and absorption enhancing effects of Glycofurol 75 and sodium glycocholate in monolayers of human intestinal epithelial (Caco-2) cells, Int. J. Pharm. 95 (1993) 209–217.
[49] J.A. Champion, Y.K. Katare, S. Mitragotri, Particle shape: a new design parameter for micro- and nanoscale drug delivery carriers, J. Controlled Release 121 (2007) 3–9.