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Research paper

Influence of preparation conditions and heat treatment on the properties of supercooled smectic cholesteryl myristate nanoparticles

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Abstract

Colloidal dispersions of cholesterol esters in the supercooled smectic state are under investigation as a novel drug carrier system in particular with respect to parenteral application. In the present study, suitable conditions for the homogenization of cholesteryl myristate dispersions stabilized with a phospholipid/bile salt blend were evaluated. For effective particle size reduction homogenization with high pressure and at temperatures above the melting temperature of the cholesterol ester (isotropic melt) is necessary. Homogenization at lower temperature where the matrix lipid is in the smectic state is less effective even when applying the highest homogenization pressure possible but still leads to dispersions with particles in the colloidal size range.

Since sterility is required for parenteral medications and is usually achieved by autoclaving for aqueous systems, the physical and chemical stability of cholesteryl myristate nanoparticles stabilized with different surface active agents during heat treatment was investigated as well. The dispersions were characterized by particle size and zeta potential measurements, differential scanning calorimetry (DSC) and high performance thin layer chromatography (HPTLC). The results indicate that cholesteryl myristate nanoparticles stabilized with phospholipid/sodium glycocholate, polyvinyl alcohol, poloxamer and poloxamine can be sterilized by autoclaving. Compared to cholesterol ester free dispersions of phospholipids, the phospholipid seems to be more stable against hydrolysis during prolonged heat treatment in the phospholipid/bile salt containing cholesteryl myristate dispersions.

Keywords: Lipid nanoparticles; Supercooled smectic nanoparticles; Homogenization; Autoclaving; Stability; Cholesterol esters

1. Introduction

Pharmaceutical drugs often show a low solubility in aqueous environments. To ensure a sufficient bioavailability or to facilitate the parenteral administration of such drugs, the use of suitable solvent mixtures or drug carriers is required. Lipid based systems like colloidal fat emulsions [1,2] or solid lipid nanoparticles [3,4] seem to be favorable carrier systems in particular with respect to physiological compatibility because they can be prepared solely on the basis of physiological substances. This also applies to supercooled smectic nanoparticles, which were introduced recently as a potential novel lipidic drug carrier system for poorly water soluble, lipophilic drugs [5]. These systems, based on a strongly supercooled thermotropic mesophase of cholesterol esters as, e.g., cholesteryl myristate, might be advantageous in comparison to colloidal fat emulsions and solid lipid nanoparticles because of the special features of the liquid crystalline state. The high viscosity of the smectic phase may lead to pronounced immobilization of incorporated drug molecules and to high physical stability of the nanoparticles. On the other hand, fluidity of the mesophase on the molecular level should allow for a higher drug load compared to the highly ordered crystalline state as present in solid lipid nanoparticles.

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Colloidal lipid dispersions can be prepared by high-pressure homogenization [6–8]. The homogenization of lipids, which are solids at room temperature such as cholesterol esters, is usually carried out at temperatures above the melting point of the lipid (high-pressure melt homogenization [3–5]). Since no organic solvents are needed for the preparation of colloidal lipid dispersions by high-pressure melt homogenization, this preparation method is especially favorable for parenteral formulations. In the present study, the applicability of two different homogenization principles using the continuously working Microfluidizer (interaction chamber [7,9]) and a discontinuously working Micron-Lab (piston-gap [8,9]) for the preparation of smectic cholesteryl myristate nanoparticles was compared. The influence of different homogenization conditions like homogenization pressure, temperature and time and/or cycle on the particle size of dispersions stabilized with a phospholipid/bile salt mixture was investigated. A small mean particle size as well as a limited amount of particles with sizes in the μm-range is a prerequisite for intravenous drug delivery [10]. An influence of the particle size on the fate of the smectic lipid nanoparticles after injection is likely as it was shown, e.g., for colloidal fat emulsions and liposome dispersions [11,12]. Furthermore, a small particle size seems to be preferable with respect to the stability of the supercooled smectic state of the cholesteryl myristate matrix in particular when the particles are stabilized on the basis of phospholipids. For such smectic nanoparticles a strong dependence of the nanoparticle recrystallization tendency during storage on the particle size was observed [13].

Because dispersions of supercooled smectic nanoparticles are of particular interest for parenteral administration sterility of the formulations is an important point. The common method for the sterilization of aqueous systems is autoclaving. Investigations on the stability of colloidal fat emulsions and solid lipid nanoparticles appear promising but showed that stability upon autoclaving is influenced by the matrix lipid beside the stabilizer system [14–20]. Sterilization is also possible by filtration of the formulation through a 0.2 μm membrane filter. However, this method is less safe and can only be used for solutions or colloidal dispersions with appropriately small particles [21]. The physical and chemical stability of cholesteryl myristate dispersions prepared with different stabilizers (phospholipid/bile salt mixture, poloxamer, poloxamine and polyvinyl alcohol) upon autoclaving was investigated by particle size and zeta potential measurements, DSC and thin layer chromatography. Since phospholipids are sensitive to hydrolysis in aqueous systems and since an excess of lyso-derivatives and free fatty acids may lead to undesired side effects after parenteral administration [22,23], a phospholipid containing cholesteryl myristate dispersion was exposed for extended times to elevated temperatures to get deeper insight into the stability under thermal stress conditions. For comparison, pure aqueous phospholipid dispersions (without cholesteryl myristate) were also investigated with respect to phospholipid hydrolysis after heat treatment.

2. Materials and methods

2.1. Materials

Cholesteryl myristate (CM, Sigma, ICN), soybean phospholipids (PL) Lipoid S100 (S100), Lipoid S75 (S75), egg yolk phospholipid Lipoid E80 (E80, Lipoid KG, D-Ludwigshafen), sodium glycocholate (SGC, Sigma), polyvinyl alcohol (PVA, Mowiol 3-83, Clariant, D-Frankfurt/Main), poloxamer 188 (POL, Lutrol F68) and poloxamine (TET, Tetronic 908, BASF, D-Ludwigshafen), thiomersal (Caesar & Loretz, D-Hilden), glycerol (Solvay, D-Rheinberg), water for injection Ph.Eur. (prepared by successive filtration, deionization, reverse osmosis and distillation), chloroform (⩾99.8%, Fluka, CH-Buchs), methanol (⩾99.9%), *n*-hexane (⩾98%) and potassium chloride (⩾99.5%, Roth, D-Karlsruhe), *n*-propanol (⩾99.8%), diethyl ether (⩾99.5%) and acetic acid (99.9%, Merck, D-Darmstadt), methyl acetate (⩾99%, Merck-Suchardt, D-Hohenbrunn). All materials were used as received.

2.2. Preparation of the dispersions

The dispersions contained 5% cholesteryl myristate (related to the whole dispersion before homogenization, all concentrations are w/w) as matrix lipid and different stabilizers (Table 1). The stabilizers were dissolved or dispersed in the aqueous phase containing 0.01% thiomersal and 2.25% glycerol at room temperature for at least 24 h (thiomersal was added as preservative to allow sample withdrawal upon storage under non-aseptic conditions and to avoid microbiological growth). Cholesteryl myristate was molten at approximately 95 °C and merged with the aqueous phase which had been heated to the same temperature. A crude emulsion was prepared by Ultra Turrax vortexing for 3–5 min at about 95 °C, transferred into a heated (85–90 °C) high-pressure homogenizer (Microfluidizer M110S, Microfluidics or Micron-Lab 40, APV-Gaulin D-Lübeck), and homogenized under different conditions (Table 1). Unless otherwise stated, samples were taken after each homogenization minute (Microfluidizer) or after every second homogenization cycle (Micron-Lab). After filtration (cellulose acetate, pore size 0.2 or 5 μm, Braun-Melsungen) and cooling to room temperature, the dispersions were stored at 23 °C. One cholesteryl myristate dispersion (dispersion G) was stored for 24 and 48 h at 90 °C in a drying heater (WTB drier, Binder, D-Tuttlingen).

Pure phospholipid dispersions were prepared by equilibrating 10 mg of the lipid (Lipoid S100, Lipoid S75 or Lipoid E80, respectively) in 1 ml water for injection for about 24 h at room temperature under manual shaking from time to time. The pure phospholipid dispersions were then stored for 24 h at 40 or 80 °C in a drying heater (WTB drier, Binder, D-Tuttlingen).

Table 1
Composition, homogenization conditions and particle size (PCS) after preparation of the dispersions

Code	Stabilizer(s)	Homogenization conditions	Particle size (PCS)	
			z-Average (nm)	PDI
Microfluidizer				
A	3.2% S100 0.8% SGC	1 min at 700 bar and 85–90 °C, then in 1 min steps at 800, 900, 1000, 1100, 1200, 1300 bar and 50–60 °C	185	0.14
B	3.2% S100 0.8% SGC	1 min at 700 bar and 85–90 °C, then in 1 min steps at 1300, 1200, 1100, 1000, 900, 800 bar and 50–60 °C	159	0.14
C[a]	3.2% S100 0.8% SGC	1 min at 700 bar and 85–90 °C, then 6 min at 1200–1300 bar and 50–60 °C	129	0.13
D	3.2% S100 0.8% SGC	In 1 min steps at 700, 800, 900, 1000, 1100, 1200, 1300 bar and 85–90 °C	96	0.15
E[a]	3.2% S100 0.8% SGC	In 1 min steps at 700, 1300, 1200, 1100, 1000, 900, 800 bar and 85–90 °C	94	0.14
F	3.2% S100 0.8% SGC	1 min at 700 bar, then 5 min at 1200–1300 bar and 85–90 °C	99	0.14
G[b]	3.2% S100 0.8% SGC	5 min at 1100 bar and 75–90 °C	109	0.13
H[a]	5% PVA	5 min at 1000–1200 bar and 80–85 °C	126	0.14
I[a]	4% TET	5 min at 1100–1200 bar and 80–85 °C	152	0.14
K[a]	4% POL	3 min at 900 bar and 82–84 °C	164	0.11
Micron-Lab				
L	3.2% S100 0.8% SGC	10 cycles at 1300 bar and 85–90 °C	183	0.13
M	3.2% S100 0.8% SGC	10 cycles at 1400 bar and 85–90 °C	153	0.14
N	3.2% S100 0.8% SGC	10 cycles at 1580 bar and 85–90 °C	144	0.15

The dispersions contain 5% CM as matrix lipid. All concentrations are w/w and related to the whole dispersion before high-pressure homogenization.
[a] Dispersions were autoclaved after preparation (C and E) or after storage (H–K). The particle size (PCS) before autoclaving of the latter was 129 nm (H), 148 nm (I) and 164 nm (K) with PDI values between 0.13 and 0.14, respectively.
[b] A fraction of the dispersion was stored for 24 and 48 h at 90 °C after preparation.

2.3. Autoclaving

Selected dispersions (Table 1) were sterilized in an autoclave with automatic control (Thema 3, Fedegari Autoclavi, I-Albuzzano) under standard conditions (121 °C, 3 bar, 15 min, 5 min compensation time, steam–air-mixture protocol). The temperature was controlled by several Pt-100-sensors, which were placed at different positions inside the autoclave. One sensor was placed in an open water-filled reference vial of the same volume and size as the sample vials. The dispersions were autoclaved directly after preparation (phospholipid based dispersions) or after storage (polymer stabilized dispersions).

2.4. Particle size and particle size distribution

Photon correlation spectroscopy (PCS) – Dynamic light scattering was measured at 25 °C and 90° with a Zeta Plus instrument (Brookhaven Instruments) after diluting the samples with purified, filtered (Sterifix 0.2 μm, Braun-Mel-sungen) water. Each sample was measured for 30–40 min (corresponding to 6–8 runs over 5 min per measurement). The intensity weighted mean diameter (*z*-average diameter) and the polydispersity index (PDI) – an indication for the width of the particle size distribution – were established using the instrument's cumulant analysis software. Laser diffraction (LD-PIDS).

The combination of laser diffraction with the PIDS (polarization intensity differential scattering) technology (LS 230 Particle Sizer, Beckman-Coulter) allows measurements over a particle size range from about 40 nm up to 2000 μm [24]. Data of laser diffraction and PIDS are combined to calculate the particle size distribution by the Coulter LS software (Beckman-Coulter). Samples were measured in purified water. Size distributions were calculated applying the Mie theory with refractive indices of 1.45 (particles) and 1.330 (dispersant, water) and a particle shape factor of one. The values given are averages of eight single runs per sample with a duration of 90 s per run.

Size measurements were only done on single samples drawn from the dispersions since such measurements have proven to be highly reproducible in this and former studies. For example, the variations (standard deviations) in the PCS size parameters of the non-autoclaved dispersions C, E ($n = 10$), H, I and K ($n = 5$) were $\leqslant$2.5 nm for the z-average and $\leqslant$0.03 for the polydispersity index over about one year of storage. Also variations between the three samples taken after 1 min of homogenization at 700 bar and 80–90 °C (Microfluidizer, dispersions D, E and F) were only minor: PCS: z-average 198.8 ± 4.7 nm, polydispersity index 0.13 ± 0.005; LD-PIDS: mean 197.3 ± 3.2 nm and median 184.7 ± 4.7 nm, D99-diameter 450.7 ± 7.2 nm ($n = 3$).

Both PCS and LD-PIDS are not suitable to detect a small number of particles in the μm-range. For this reason, selected dispersions were investigated for the presence of larger particles and aggregates, respectively, by light microscopy (DMRXP, Leica, D-Wetzlar). The samples were viewed with 400 and 1000 fold magnification in the differential interference contrast mode.

2.5. Differential scanning calorimetry (DSC)

DSC measurements were made on a Pyris-1 calorimeter (Perkin-Elmer). Approximately 8–15 mg samples were accurately weighed into standard aluminum pans. An empty pan was used as reference. The samples were heated to 90 °C, cooled to −10 or −13 °C and heated again to 90 °C with a scan rate of 5 °C/min. Between all temperature scans, isothermal steps with a duration of 5 min were inserted. All DSC curves shown in Fig. 3 are normalized to a sample weight of 1 mg. For better visualization, the DSC curves are shifted along the ordinate.

2.6. Zeta potential

Zeta potential measurements were made with a Zetasizer ZS (Malvern Instruments) at 25 °C. The dispersions were diluted with sterile-filtered TRIS-buffer (10 mM, pH 7.4) and filled into special cuvettes (DTS 1060 disposable zeta cell, Malvern instruments). The zeta potential values were calculated by the DTS Dispersion Technology Software (Version 3.30 2002, Malvern instruments) using the Smoluchowski model. For simplification, the values of pure water (viscosity 0.8872 mPa*s, refractive index 1.330 and dielectric constant 79) were used for the dispersion medium (water phase) for calculations. The values given are averages of four measurements with 30 single runs each.

2.7. High performance thin layer chromatography (HPTLC)

The investigations are based on a method described by Yao et al. [25]. HPTLC plates (10 × 10 cm, nano SILGUR-20, Macheray-Nagel, D-Düren) with combined layers of silica gel (application and pre-concentration zone) and nano silica gel 60 (separation zone) were used. Before use, the TLC plates were developed with solvent mixture A (Table 2) and heated to 110 °C for 10 min. 50 μl of dispersion or 10 mg lipid was dissolved in 450 or 1000 μl of a mixture of chloroform and methanol (50:50 v/v), respectively. Nine to eleven samples of the resulting solutions were placed pointwisely in the lower pre-concentration zone using a μl-syringe (2 μl, Hamilton, CH-Bonaduz). The plates were developed with solvent mixture A until a height of 4.6 cm above the pre-concentration zone was reached. This procedure was followed by complete development with solvent mixture B (Table 2) and finally with pure n-hexane. Before placing the plates in a new chromatography chamber, the plates were dried carefully under a stream of warm air. The lipid spots were detected by dipping the plates into a staining solution (75 g $CuSO_4 \cdot 5H_2O$, 100 ml phosphoric acid 85%, 900 ml purified water) for about three seconds and heating to 170 °C for 7–8 min. For the identification of the lipid spots, a solution of known composition (kindly provided by the IMB Jena) containing sphingomyelin, phosphatidylcholine, phosphatidylinositol, phosphatidylglycerol, sulfogalactosylceramide, galactosylceramide, monoglyceride, cholesterol, fatty acid, triglyceride, cholesterol ester and squalene as well as solutions of the pure lipids used for the preparation of the dispersions was used. For the semi-quantitative analysis, the HPTLC plates were scanned and the images were analyzed densitometrically using the Scion-Image software (Scion Corporation 2000). HPTLC analysis was done in triplicate for all samples. The linearity of the method was checked in the range of interest with solutions of pure phospholipid.

Table 2
Composition of the solvents used for HPTLC

Solvent mixture	Function	Composition (volume parts)
A	Separation of polar lipids as phospholipids, mono- and diglycerides	26.6 Methyl acetate 26.6 n-Propanol 26.6 Chloroform 10.6 Methanol 9.6 KCl solution 0.25% (w/v)
B	Separation of nonpolar lipids as triglycerides and cholesterol esters	75.0 n-Hexane 23.0 Diethyl ether 2.0 Water-free acetic acid
C	Optimization of separation	100 n-Hexane

3. Results

3.1. Influence of homogenization conditions

To study the influence of the homogenization conditions dispersion preparation was always based on the

same composition (5% CM, 3.2% S100, 0.8% SGC). Due to the high smectic-isotropic phase transition temperature of cholesteryl myristate (about 85 °C in the bulk state [5,26]), melt homogenization has to be carried out at relatively high temperatures in order to assure the isotropic state of the lipid melt. Since this may induce chemical degradation of temperature sensitive components, it was investigated if homogenization at lower temperatures corresponding to the smectic state of cholesteryl myristate (50–60 °C) would be effective as well. However, a first homogenization step at high temperature was always required to transfer all particles into the colloidal state and to prevent crystallization of the matrix lipid in the storage vessel of the homogenizer.

3.1.1. Homogenization with the Microfluidizer

PCS *z*-average diameters below 100 nm were obtained for all dispersions homogenized at a temperature of around 85 °C after the full homogenization procedure (dispersions D–F, Fig. 1). The application of high-pressure during the first homogenization minutes was most effective and resulted in dispersions with *z*-average diameters of around 100 nm already after the 4th or 5th homogenization minute, whereas a comparable particle size was only obtained after about the 6th homogenization minute when the pressure was stepwisely increased from 700 to 1200 bar. In dispersions homogenized at 85–90 °C a slight increase of the PDI was observed over the last homogenization minutes. However, the PDI values were between 0.12 and 0.15 for

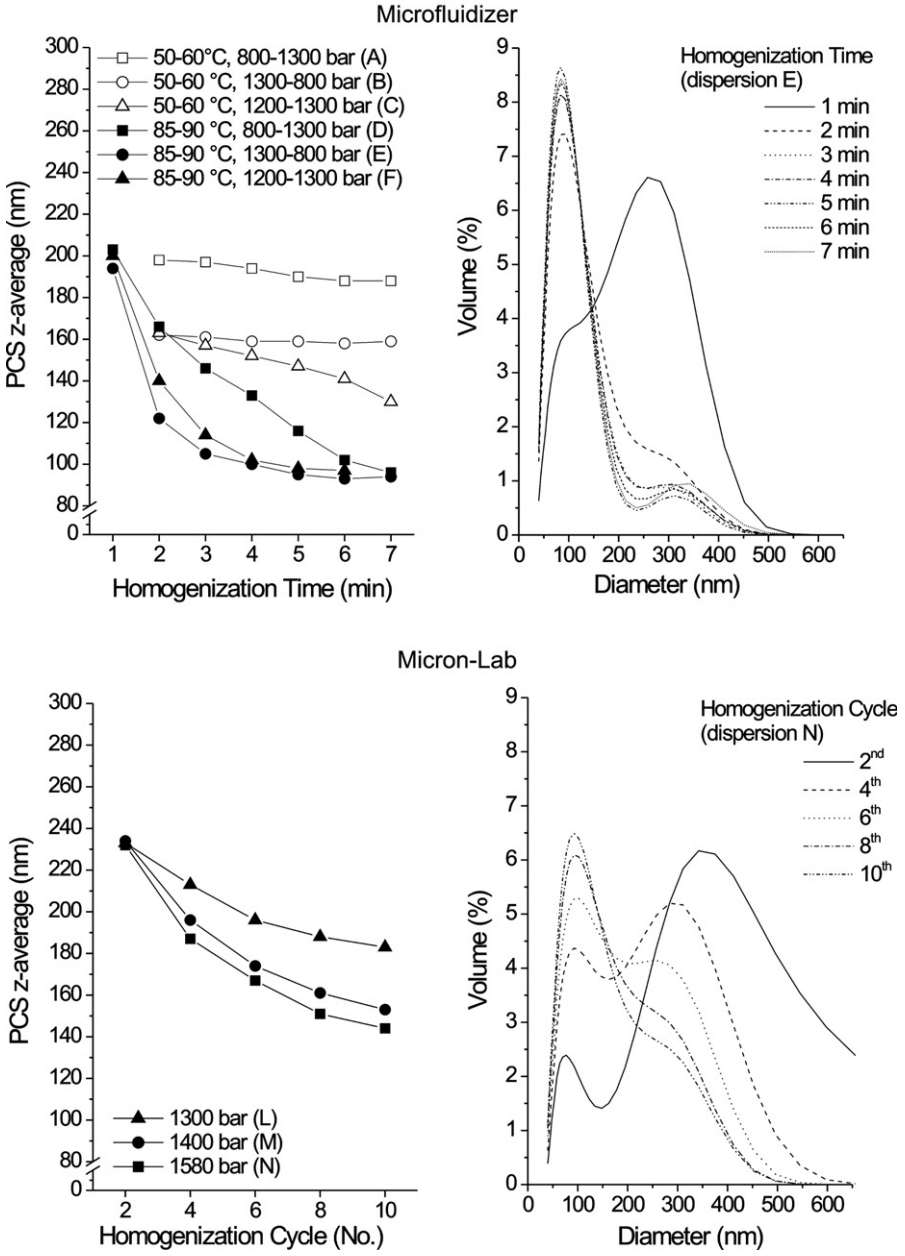

Fig. 1. Particle size (PCS, left) and particle size distribution (LD-PIDS, right, dispersion E and N, respectively) in dependence on homogenization time. Top: Microfluidizer. Bottom: Micron-Lab.

all dispersions throughout the homogenization process. For the dispersions, for which the homogenization temperature was decreased from initially 85 °C to about 50–60 °C after the 1st homogenization minute (dispersions A–C), no samples were taken after the 1st homogenization minute. However, since the conditions were the same as for dispersions D–F, the particle sizes should be comparable after the 1st homogenization minute. Homogenization at this lower temperature (50–60 °C), where the cholesteryl myristate particles are in the highly viscous smectic state, required the application of high homogenization pressure over at least six minutes to achieve a distinct reduction of the particle size (dispersion C). For both other dispersions (A and B), the decrease of particle size after the 2nd homogenization minute was only slight (about 10 (A) and 3 (B) nm, respectively, Fig. 1). The polydispersity indices were between 0.12 and 0.14 and were not distinctly influenced by homogenization time.

The results of the LD-PIDS measurements were in good agreement with data obtained by PCS. For all dispersions a bimodal particle size distribution was measured. The fraction of large particles decreased with increasing homogenization time, particularly for dispersions homogenized at higher temperatures as exemplified for dispersion E in Fig. 1. Already after the first homogenization minute, no particles in the μm-range were detected (D99-values <500 nm for dispersions E–F).

3.1.2. Homogenization with the Micron-Lab

Colloidal dispersions could also be obtained by homogenization with the Micron-Lab. In spite of comparable thermal conditions and higher homogenization pressures the final mean particle sizes were, however, larger than after homogenization with the Microfluidizer (Fig. 1). After 10 cycles of homogenization the mean particle sizes were in the range between 140 and 160 nm when homogenization pressures of 1400 and 1580 bar were applied. With increasing number of homogenization cycles, the PCS polydispersity indices decreased slightly. The polydispersity indices were between 0.16 and 0.18 after the 2nd and between 0.15 and 0.13 after the 10th homogenization cycle. Particles in the μm-range were still detected by LD-PIDS measurements after the 2nd homogenization cycle in all dispersions (D99-values between 1.8 and 2.0 μm). However, after the 4th homogenization cycle, particles in the μm-range were not observed any longer (D99-values <600 nm). The LD-PIDS particle size distributions of the final dispersions were bimodal as for the dispersions homogenized with the Microfluidizer exemplified for dispersion N in Fig. 1. The volume fraction at higher diameters was, however, larger for the dispersions prepared with the Micron-Lab.

3.2. Stability upon autoclaving and heat treatment

Selected dispersions with different stabilizers and *z*-average diameters between around 100 and 200 nm were autoclaved directly after high-pressure homogenization or after storage for several months (Table 1). Storage prior to autoclaving did not cause changes in particle size and particle size distribution (Table 1). Moreover, one dispersion stabilized with the phospholipid/bile salt mixture (dispersion G) was stored for 24 and 48 h at 90 °C to obtain information about the physical and chemical stability under thermal stress conditions.

No changes of the macroscopic appearance and the mean particle size were observed for dispersions stabilized with the phospholipid/bile salt mixture or with PVA after autoclaving (Fig. 2). Even storage at 90 °C for 24 and 48 h did not cause physical instability of a phospholipid containing dispersion; the *z*-average diameter of this dispersion was 109 nm before and 111 nm after heat treatment for 48 h. However, a yellowish discoloration was observed with increasing duration of heat treatment. In contrast, autoclaving led to a distinct increase of the particle size of the dispersions stabilized with poloxamer and poloxamine (Fig. 2). Upon storage of the autoclaved dispersions, no further increase of the particle size was observed in PCS and LD-PIDS measurements. Furthermore, light microscopic images of the autoclaved dispersions did not differ distinctly from those of the non-autoclaved ones.

While the zeta potential of the phospholipid/bile salt stabilized dispersions was only slightly increased after autoclaving (by about 6 mV), longer heat treatment led to a distinct increase of the zeta potential from initially −30 to −43 mV after storage for 48 h at 90 °C. The increase in zeta potential might be caused by phospholipid hydrolysis whereupon lyso-phosphatidylcholine and free fatty acids are formed. No changes of the zeta potential were measured for the dispersions stabilized with polymers. The values were between −2 and −4 mV for all these dispersions before and after autoclaving.

Autoclaving did not lead to distinct alterations of the phase behavior in DSC measurements of dispersions stabilized with the phospholipid/bile salt blend and PVA as

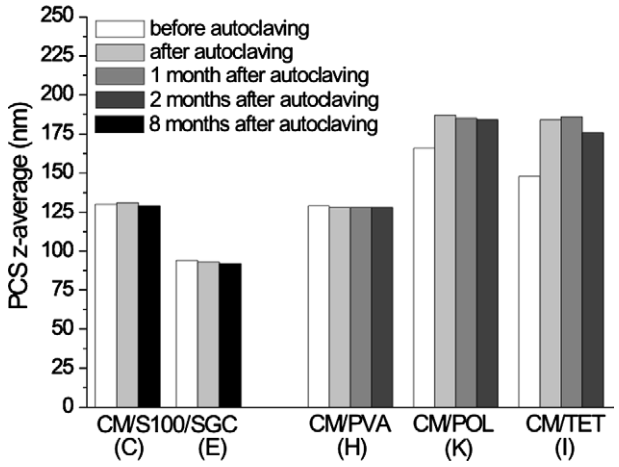

Fig. 2. PCS *z*-average before and after autoclaving as well as after storage of the autoclaved dispersions. The PDI were ⩽0.15 for all dispersions.

shown for the crystallization events in Fig. 3. However, longer heat treatment led to an altered crystallization behavior of the dispersion stabilized with the phospholipid/bile salt mixture. With increasing duration of storage at 90 °C, the main crystallization event at lower temperature shifted to lower temperatures (Fig. 3). The area of the crystallization event at higher temperature decreased with increasing thermal stress. The liquid crystalline phase transitions as well as the melting behavior were only slightly altered after heat treatment. Similar changes of the crystallization pattern were observed for phospholipid-stabilized dispersions upon long-term storage [13]. In dispersions stabilized with poloxamer and poloxamine, the phase behavior was slightly changed after autoclaving. The phase transitions were sharpened and, e.g., the crystallization event was shifted to higher temperatures (Fig. 3).

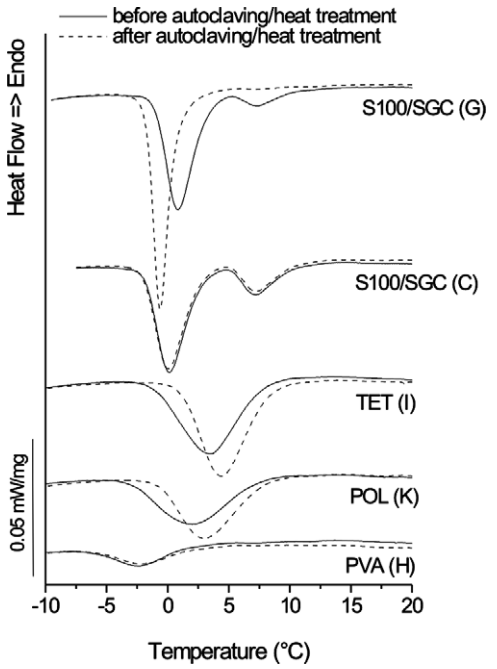

Fig. 3. DSC cooling curves (5 °C/min) of the samples before and after autoclaving (dispersions C, I, H and K) and before and after thermal stress (dispersion G, storage at 90 °C for 48 h).

No distinct changes in the composition of dispersions stabilized with polymers were found by HPTLC after autoclaving. For phospholipid containing dispersions already high-pressure melt homogenization led to a small degree of phospholipid hydrolysis as indicated by the presence of free fatty acids (FFA) and lyso-phosphatidylcholine (Lyso-PC, Fig. 4). Phospholipid hydrolysis was increased after autoclaving, but only severe thermal stress (storage at 90 °C for 48 h) led to distinct phospholipid hydrolysis (Fig. 4). However, compared with the pure phospholipid dispersions, in which already storage for 24 h at 80 °C led to a massive hydrolysis of the phospholipids, chemical degradation was less pronounced in the cholesteryl myristate dispersion (Figs. 4 and 5).

4. Discussion

For the preparation of colloidal cholesteryl myristate dispersions with small particle sizes, the continuously working Microfluidizer seems to be more suitable than the use of the Micron-Lab. Homogenization was most efficient when carried out at high temperature (>85 °C) and applying high pressures (>1000 bar) for 3–5 min. For dispersions stabilized with the phospholipid/bile salt blend, a PCS *z*-average of about 100 nm could be obtained under these conditions. However, longer homogenization did not lead to a further particle size reduction of the phospholipid-bile salt stabilized dispersions. Particularly due to the discontinuous process, the efficiency of the homogenization using the Micron-Lab was lower compared with microfluidization even when the highest possible homogenization pressure (1580 bar)

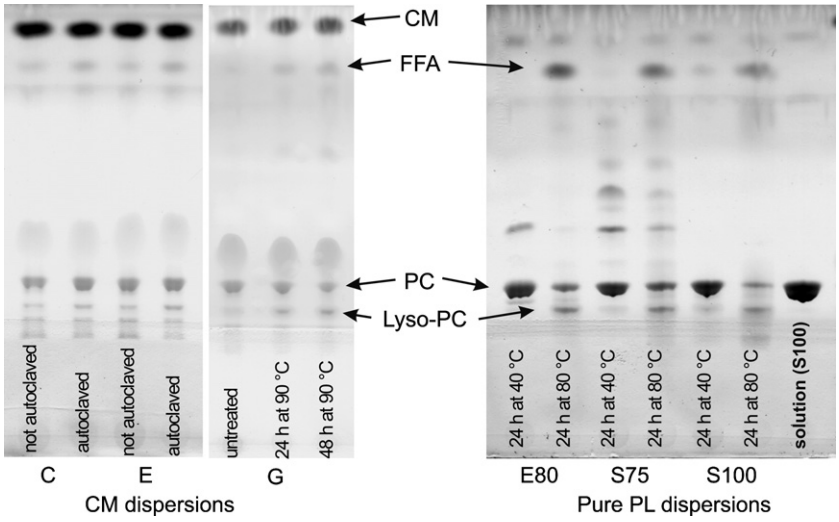

Fig. 4. Selected results of HPTLC investigations of CM dispersions stabilized with the phospholipid/bile salt mixture (left) and pure phospholipid dispersions (right). The solution (S100) refers to the solution of the phospholipid in chloroform/methanol.

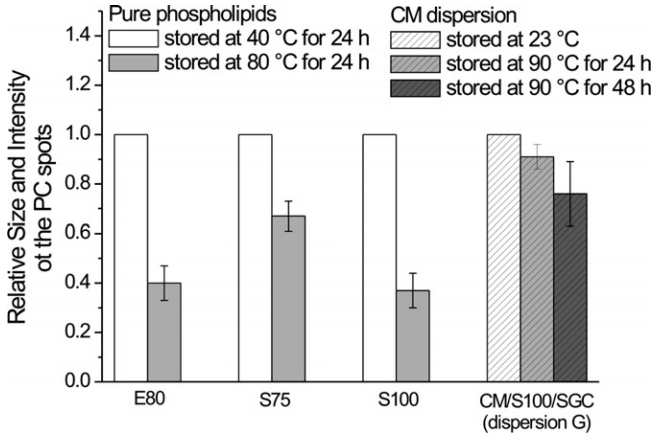

Fig. 5. Semi-quantitative analysis of the degree of phospholipid hydrolysis: decrease of the amounts of phosphatidylcholine (PC) in dependence on thermal treatment. The PC amount found at storage at 40 °C (pure PL dispersions) or 23 °C (CM dispersion) was used as reference value.

was applied. However, the results of both homogenization procedures cannot be compared directly, because the effective homogenization time was much shorter using the discontinuously working Micron-Lab. At least 10 homogenization cycles are necessary for an effective homogenization time of about two minutes. Taking this into account, homogenization efficiency was comparable for both homogenizers (*z*-average 140 nm for dispersion F after the 2nd homogenization minute and 144 nm for dispersion N after 10 homogenization cycles). Moreover, the results of the particle size measurements (Fig. 1, panels to the left) indicate that a further reduction in particle size may be possible by continuation of the homogenization process with the Micron-Lab (in particular for dispersions M and N). In contrast, a plateau in the particle size versus homogenization time curve was obtained for homogenization with the Microfluidizer after about the 5th minute when the highest homogenization pressure was applied and homogenization was carried out at temperatures above the melting temperature of the matrix lipid (dispersions E and F). However, for practical reasons, in particular because of loss of formulation during each homogenization cycle, the applicable number of homogenization cycles with the Micron-Lab is limited. Furthermore, the thermal stress (holding the dispersions at 90 °C over the whole homogenization time including the unavoidable breaks in between the homogenization cycles) is more pronounced upon homogenization with the discontinuously working Micron-Lab compared to the continuous homogenization using the Microfluidizer.

Among other parameters like the homogenization pressure, the efficiency of homogenization depends on the viscosity of the dispersed phase [14,27]. Therefore, homogenization at lower temperatures (50–60 °C), where the particles are in the smectic state, was much less efficient due to the high viscosity of the smectic phase [5,26]. A distinct size reduction could only be obtained when the highest possible homogenization pressure was applied for longer times (7 min).

Autoclaving did not lead to a physical instability of the investigated colloidal dispersions. The increase in particle size observed for the dispersions stabilized with poloxamer and poloxamine after autoclaving can probably be attributed to a partial destabilization caused by clouding of the polymer at this high temperature. This might lead to fusion of particularly smaller particles. The slight alterations in the phase behavior observed in DSC measurements are in good agreement with this assumption. Instability upon autoclaving of triglyceride dispersions stabilized with poloxamer has been described in the literature with a strong dependence of the degree of destabilization on the type of the lipid matrix [20,28].

Concerning the chemical stability, phospholipid hydrolysis was only slightly increased by autoclaving of the cholesteryl myristate dispersions, which is in agreement with results of studies on autoclaving of liposome dispersions with pH 7.4 [29] and colloidal fat emulsions [30] where no distinct hydrolysis was detected. The high-pressure melt homogenization process led to a slight phospholipid hydrolysis because of the high temperature necessary over the whole process (pre-dispersion and homogenization) and probably also due to the high energy input upon high-pressure homogenization as it was shown for liposome preparations by homogenization using a Microfluidizer [31]. Even severe thermal stress (48 h at 90 °C) did, however, not lead to a massive phospholipid hydrolysis although the degree of hydrolysis increased with the duration of thermal stress. Phospholipid hydrolysis follows first order kinetics as has been shown for pure phospholipid dispersions [32] as well as for colloidal fat emulsions [30,33]). In the present study, phospholipid hydrolysis was more pronounced in pure phospholipid dispersions (although these systems were stored at only 80 °C for 24 h) than in the cholesterolyl myristate containing dispersions. This result suggests that phospholipid molecules located in liposomes, which are present in the dispersions due to the excess of stabilizers as observed in electron microscopic investigations [5], might be more sensitive to hydrolysis than those located in the interface of the lipid nanoparticles. It should be mentioned that the formation of free fatty acids due to hydrolysis will lead to a decrease in pH [22,34,35] which further promotes phospholipid hydrolysis [29,32]. The pH in dependence on thermal treatment was not investigated in the present study and needs, therefore, further evaluation.

In the present study, we used thin layer chromatography for a first evaluation of chemical degradation upon autoclaving and thermal stress because of rapidity and low demand of equipment for this method. For quantitative analysis HPLC is commonly used but usually requires special equipment for the adequate detection of the lipids [33].

In conclusion, the results of the present study demonstrate that colloidal dispersions of cholesteryl myristate with particle sizes suitable for parenteral administration can be prepared by established methods like high-pressure melt homogenization. Furthermore, the dispersions can be sterilized by autoclaving without remarkable loss of quality.

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References

[1] Y.A. Carpentier, I.E. Dupont, Advances in intravenous lipid emulsions, World J. Surg. 24 (2000) 1493–1497.
[2] S. Klang, S. Benita, Design and evaluation of submicron emulsions as colloidal drug carriers for intravenous administration, in: S. Benita (Ed.), Submicron emulsions in drug targeting and delivery, Harwood Academic Publishers, Amsterdam, Netherlands, 1998, pp. 119–152.
[3] W. Mehnert, K. Mäder, Solid lipid nanoparticles: production, characterization and applications, Adv. Drug Deliv. Rev. 47 (2001) 165–196.
[4] H. Bunjes, B. Siekmann, Manufacture, characterization, and application of solid lipid nanoparticles in drug delivery systems, in: S. Benita, J. Swarbrick (Eds.), Microencapsulation: Methods and Industrial Applications, Taylor & Francis, New York, USA, 2006, pp. 213–268.
[5] 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.
[6] A.G. Floyd, S. Jain, Injectable emulsions and suspensions, in: H.A. Liebermann (Ed.), Pharmaceutical Dosage Forms: Disperse Systems, vol. 2, Marcel Dekker, New York, USA, 1996, pp. 261–318.
[7] C. Washington, S.S. Davis, The production of parenteral feeding emulsions by Microfluidizer, Int. J. Pharm. 44 (1988) 169–176.
[8] T. Bock, P. Kleinebudde, B.W. Müller, Manufacture of emulsions by means of high-pressure homogenisation: influence of homogenization parameters, oils and surfactants, in: R.H. Müller, S. Benita, B. Böhm (Eds.), Emulsions and Nanosuspensions for the Formulation of Poorly Soluble Drugs, Medpharm Scientific Publishers, Stuttgart, Germany, 1998, pp. 201–236.
[9] V. Naini, S.K. Singh, Homogenization and homogenizers, in: J. Swarbrick, J.C. Boylan (Eds.), Encyclopedia of Pharmaceutical Technology, Marcel Dekker, New York, USA, 2002, pp. 2458–2480.
[10] S.H. Yalkowsky, J.F. Krzyzaniak, G.H. Ward, Formulation-related problems associated with intravenous drug delivery, J. Pharm. Sci. 87 (1998) 787–796.
[11] O. Lutz, Z. Meraihi, J.L. Mura, A. Frey, G.H. Riess, A.C. Bach, Fat emulsion particle size: influence on the clearance rate and the tissue lipolytic activity, Am. J. Clin. Nutr. 50 (1989) 1370–1381.
[12] R.L. Juliano, D. Stamp, The effect of particle size and charge on the clearance rates of liposomes and liposome encapsulated drugs, Biochem. Biophys. Res. Commun. 63 (1975) 651–658.
[13] J. Kuntsche, M.H.J. Koch, M. Drechsler, H. Bunjes, Crystallization behavior of supercooled smectic cholesteryl myristate nanoparticles containing phospholipids as stabilizers, Colloids Surf. B 44 (2005) 25–35.
[14] M. Jumaa, B.W. Müller, The effect of oil components and homogenization conditions on the physicochemical properties and stability of parenteral fat emulsions, Int. J. Pharm 163 (1998) 81–89.
[15] M. Jumaa, B.W. Müller, Development of a novel parenteral formulation for tetrazepam using a lipid emulsion, Drug Dev. Ind. Pharm. 27 (2001) 1115–1121.
[16] T. Yamaguchi, K. Nishizaki, S. Itai, H. Hayashi, H. Ohshima, Physicochemical characterization of parenteral lipid emulsion: determination of Hamaker constants and activation energy of coalescence, Pharm. Res. 12 (1995) 342–347.
[17] T. Yamaguchi, K. Nishizaki, S. Itai, H. Hayashi, H. Ohshima, Physicochemical characterization of parenteral lipid emulsion: influence of cosurfactants on flocculation and coalescence, Pharm. Res. 12 (1995) 1273–1278.
[18] G. Chansiri, R.T. Lyons, M.V. Patel, S.L. Hem, Effect of surface charge on the stability of oil/water emulsions during steam sterilization, J. Pharm. Sci. 88 (1999) 454–458.
[19] R. Cavalli, O. Caputo, M.E. Carlotti, M. Trotta, C. Scarnecchia, M.R. Gasco, Sterilization and freeze-drying of drug-free and drug-loaded solid lipid nanoparticles, Int. J. Pharm. 148 (1997) 47–54.
[20] C. Schwarz, W. Mehnert, J.S. Lucks, R.H. Müller, Solid lipid nanoparticles (SLN) for controlled drug delivery. I. Production, characterization and sterilization, J. Control. Release 30 (1994) 83–96.
[21] D.M. Lidgate, T. Trattner, R.M. Shultz, R. Maskiewicz, Sterile filtration of a parenteral emulsion, Pharm. Res. 9 (1992) 860–863.
[22] J. Boberg, I. Håkansson, Physical and biological changes in an artificial fat emulsion during storage, J. Pharm. Pharmacol. 16 (1964) 641–646.
[23] D.F. Driscoll, Lipid injectable emulsions: pharmacopeial and safety issues, Pharm. Res. 23 (2006) 1959–1969.
[24] R. Xu, Improvements in particle size analysis using light scattering, in: R.H. Müller, W. Mehnert (Eds.), Particle and Surface Characterizing Methods, Scientific Publisher, Stuttgart, Germany, 1997, pp. 27–56.
[25] J.K. Yao, G.M. Rastetter, Microanalysis of complex tissue lipids by high-performance thin-layer chromatography, Anal. Biochem. 150 (1985) 111–116.
[26] K. Sakamoto, R.S. Porter, J.F. Johnson, The viscosity of mesophases formed by cholesteryl myristate, in: G.H. Brown (Ed.), Liquid Crystals 2 Part II, Gordon and Breach Science Publisher, New York, USA, 1969, pp. 237–249.
[27] S. Jahnke, The theory of high-pressure homogenization, in: R.H. Müller, S. Benita, B.H.L. Böhm (Eds.), Emulsions and Nanosuspensions for Formulation of Poorly Soluble Drugs, Scientific Publishers, Stuttgart, Germany, 1998, pp. 177–200.
[28] M. Jumaa, B.W. Müller, The stabilization of parenteral fat emulsion using non-ionic ABA copolymer surfactant, Int. J. Pharm. 174 (1998) 29–37.
[29] N.J. Zuidam, S.S.L. Lee, D.J.A. Crommelin, Sterilization of liposomes by heat treatment, Pharm. Res. 10 (1993) 1591–1596.
[30] L. Rabinovich-Guilatt, C. Dubernet, K. Gaudin, G. Lambert, P. Couvreur, P. Chaminade, Phospholipid hydrolysis in a pharmaceutical emulsion assessed by physicochemical parameters and a new analytical method, Eur. J. Pharm. Biopharm. 61 (2005) 69–76.
[31] T. Purmann, E. Mentrup, J. Kreuter, Preparation of SUV-liposomes by high-pressure homogenization, Eur. J. Pharm. Biopharm. 39 (1993) 45–52.
[32] M. Grit, J.H. de Smidt, A. Struijke, D.J.A. Crommelin, Hydrolysis of phosphatidylcholine in aqueous liposome dispersions, Int. J. Pharm. 50 (1989) 1–6.
[33] C.J. Herman, M.J. Groves, Hydrolysis kinetics of phospholipids in thermally stressed intravenous lipid emulsion formulations, J. Pharm. Pharmacol. 44 (1992) 539–542.
[34] C.J. Herman, M.J. Groves, The influence of free fatty acid formation on the pH of phospholipid-stabilized triglyceride emulsions, Pharm. Res. 10 (1993) 774–776.
[35] C. Washington, S.S. Davis, Ageing effects in parenteral fat emulsions: the role of fatty acids, Int. J. Pharm. 39 (1987) 33–37.