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Research paper

Solvent injection as a new approach for manufacturing lipid nanoparticles – evaluation of the method and process parameters

M.A. Schubert, C.C. Müller-Goymann*

Institut für Pharmazeutische Technologie, TU Braunschweig, Braunschweig, Germany

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Abstract

Lipid nanoparticles (LNP) can be prepared by rapidly injecting a solution of solid lipids in water-miscible solvents or a water-miscible solvent mixture into water. The aim of the present study was to evaluate the potential of this method for the preparation of LNP and the physicochemical characterization of the particles produced by this method. The results show that solvent injection is a potent and versatile approach for LNP preparation. Acetone, ethanol, isopropanol and methanol are suitable solvents in contrast to ethylacetate with which no LNP could be prepared. The obtained particle sizes (z-average) were between 80 and 300 nm depending on the preparation conditions. Up to 96.5% of the employed lipid was directly transformed into LNP. The LNP formation process seems to be diffusion controlled. Physicochemical characterization of the particles by differential scanning calorimetry (DSC), transmission electron microscopy and X-ray diffraction analysis reveals a distinct decrease in crystallinity of the colloidal lipid in comparison to the bulk lipid. Furthermore, DSC analysis of LNP hints at a delayed recrystallization of the colloidal lipid and the presence of two modifications. Therefore, a certain physical instability of the LNP has to be considered.

Keywords: Solid lipid; Lipid nanoparticles; Physicochemical characterization; Solvent injection; Crystallization

1. Introduction

Nanoparticles based on solid lipids have been proposed as a promising alternative colloidal drug delivery system to polymer nanoparticles and liposomes by Westesen [1] and Müller [2]. They combine the advantages of colloidal lipid emulsions with advantages of particles with a solid matrix [3]. Consisting of physiological and biodegradable lipids, lipid nanoparticles (LNP) are suitable for the incorporation of lipophilic, hydrophilic and poorly water soluble drugs within the lipid matrix in considerable amounts [4,5]. Usually lipids such as dynasan®, witepsol®, compritol® and cetyl palmitate are used as matrix materials.

The standard production method for the preparation of lipid nanodispersions based on solid lipids is high pressure homogenization [6]. Furthermore, LNP can be prepared by precipitation both from microemulsions and emulsions containing organic solvents [7,8]. The preparation of LNP with these methods involves several critical process parameters like high temperatures, high pressures, toxicologically problematic solvents, high emulsifier concentrations, etc. For example, heat and cavitation cause significant thermodynamical and mechanical stress for the resulting product. In contrast, high emulsifier concentrations and residual solvents are more problematic for the application. Therefore, an alternative production method which is commonly employed for the preparation of liposomes [9] and polymer nanoparticles [10] was investigated for its suitability for the preparation of LNP. This method offers clear advantages over the existing methods such as the use of pharmaceutically acceptable organic solvents, no need for high pressure homogenization, easy handling and a fast production process without technically sophisticated equipment. It is based on lipid precipitation from a dissolved lipid in solution. For this purpose, a solution of the lipid in a water-miscible solvent or a water-miscible solvent mixture is rapidly injected into an aqueous phase with or without surfactant.

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* Corresponding author. Technische Universität Braunschweig, Institut für Pharmazeutische Technologie, Mendelssohnstrasse 1, D-38106 Braunschweig, Germany. Tel.: +49-531-3915650; fax: +49-531-3918108.

E-mail address: c.mueller-goymann@tu-bs.de (C.C. Müller-Goymann).

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2. Materials and methods

2.1. Materials

Solid lipids: softisan® 100, softisan® 142, softisan® 154, witepsol® H35 (Condea, D-Witten), cetyl palmitate (Caelo, D-Hilden). Softisan® lipids consist of a triglyceride mixture of natural, saturated, even-numbered and unbranched fatty acids with a chain length from C_{10} to C_{18}. Softisan® 100 and 142 have melting points of 33.5–35.5 °C and 42–44 °C, respectively. They are also specified as 'hydrogenated coco-glycerides' whereas softisan® 154 with a melting point of 53–58 °C is specified as 'hydrogenated palm oil'. Witepsol® H35 is a half synthetic suppositories base obtained from coconut oil with a melting point of about 35 °C. In contrast, cetyl palmitate is a mixture of esters, containing C_{14}–C_{18} acids and alcohol components with a melting point of approximately 54 °C.

Emulsifiers: phospholipon® 90G (Phospholipid, D-Köln), containing at least 90% phosphatidylcholine, polysorbate 80 (Unigema, B-Everberg), poloxamer 188 (Synopharm, D-Barsbüttel).

Solvents: acetone, ethanol, ethylacetate, methanol, isopropanol, 85% glycerol (Sigma-Aldrich, D-Seelze), bidistilled water.

Devices: single-use syringe inject®, injection needle sterican® 0.40 × 12 mm (Braun, D-Melsungen), paper filter ∅ 90 mm (Schleicher & Schnell, D-Dassel).

2.2. Preparation of LNP

LNP were prepared by a modified solvent injection technique. The lipids were dissolved in a water-miscible solvent and a water-miscible solvent mixture (1—100 mg/ml) and then rapidly injected through an injection needle into a stirred (330 rev./min) aqueous phase with or without surfactant. The resulting dispersion was then filtered with a paper filter in order to remove any excess lipid.

2.3. Particle size measurement

The size distribution of the LNP was investigated by photon correlation spectroscopy (PCS) using a Zetasizer 3 (Malvern, D-Herrenberg) modified with a He/Ne laser model 127 (Spectra Physics, USA, Mt. View, CA). The different systems were investigated at an angle of 90° in a measuring cell AZ 10 tempered at 293 K. Each sample was diluted with filtered bidistilled water until the appropriate concentration of particles was achieved to avoid multiscattering events and measured with a sample time of 30 ms for 10 min in serial mode. Each measurement was performed in triplicate and both the particle z-average diameter and polydispersity index (PI) were determined.

2.4. Transmission electron microscopy (TEM)

Samples were shock-frozen in melting nitrogen at 63 K between two flat gold holders. The frozen samples were fractured at 173 K in a BAF 400 instrument (Balzers, D-Wiesbaden). Shadowing was performed with platinum/carbon (2 nm) at 45° and with pure carbon at 90° for replica preparation. After cleaning with a chloroform/methanol mixture (1:1), the replicas on uncoated grids were viewed using a transmission electron microscope EM 300 (Philips, D-Kassel).

2.5. Differential scanning calorimetry (DSC)

Measurements were performed on a calorimeter DSC 220C connected to a disc station 5200H (Seiko, J-Tokyo). Approximately 15 mg of the dispersion or 0.039 mg of bulk material were accurately weighed in an aluminium crucible and fused on cold. The probes were measured against an empty reference crucible in a temperature range of 5–75 °C at a heating rate of 5 °C/min.

2.6. Yield

The yield, which refers to the quantity of nanoparticles recovered from the preparation process, was determined gravimetrically after drying 10 ml of suspension in an oven at 30 °C until constant weight was obtained. It is expressed as the percentage ratio of the lipid amount in the suspension to the theoretical amount.

2.7. Wide angle X-ray diffraction (WAXD)

WAXD studies were performed in a Debye-Scherrer camera with a perimeter of 360 mm installed on a PW 1830 generator with a PW 2253/11 X-ray tube equipped with a copper anode and a nickel K_β filter (Philips, D-Kassel). Samples were filled into standard glass X-ray capillaries and the exposure time was varied between 24 and 48 h using Agfa D7 X-ray films. The diffraction rings were calculated using Bragg's equation.

2.8. Viscosity measurement

The dynamic viscosity of the aqueous phase was varied by the addition of 10%, 20%, 30% and 50% (v/v) glycerol. The viscosity of the aqueous phase was determined with an Ubbelohde viscometer (Schott, D-Hofheim) with an instrument constant $k = 0.009615$. Previously, the density was determined with a digital densitometer (Anton Paar, A-Graz).

3. Results and discussion

3.1. Method screening

For the preparation of LNP by solvent injection several water-miscible solvents were screened. Acetone, ethanol, isopropanol and methanol were suitable solvents in contrast to ethylacetate with which no LNP could be obtained. LNP

Table 1
Reproducibility in different solvents for softisan® 100 (three batches for each solvent with $n = 6$)

Solvents	z-Average ± SD (nm)			PI ± SD		
	Batch 1	Batch 2	Batch 3	Batch 1	Batch 2	Batch 3
Acetone	213.0 ± 1.9	226.6 ± 14.0	233.9 ± 5.2	0.202 ± 0.025	0.203 ± 0.017	0.218 ± 0.072
Ethanol	158.9 ± 15.2	163.3 ± 29.8	156.4 ± 10.2	0.340 ± 0.061	0.269 ± 0.081	0.228 ± 0.070
Isopropanol	148.5 ± 4.3	150.6 ± 2.1	152.2 ± 15.3	0.182 ± 0.007	0.159 ± 0.047	0.196 ± 0.022

preparation with methanol was not examined more closely, as the solubility of lipids in methanol was at least three times lower in comparison to the other solvents and due to the higher toxicity of the solvent. With all the studied solid lipids, i.e. softisan® 100, 142 and 154, witepsol® H35 and cetyl palmitate, it was possible to prepare nanoparticles. The addition of polysorbate 80 or poloxamer 188 as emulsifier in the aqueous phase or of phospholipon® 90G in the solvent phase leads to smaller particles. Polysorbate 80 and poloxamer 188 appear to be similarly effective in terms of particle size reduction. The addition of phospholipon® 90G as emulsifier in the organic phase leads to the smallest LNP. Since the formation of liposomes could not be excluded no further studies were conducted with phospholipon® 90G. The obtained particle size varied between 80 and 300 nm and was dependent on the preparation conditions such as lipid concentration, injected amount, emulsifier concentration, and solvent. The effects of these process parameters will be discussed in detail in Sections 3.2 and 3.3.

3.2. Assessment of reproducibility

After successful screening of the 'solvent injection' method for nanoparticle preparation the reproducibility, reliability and efficiency of the method were assessed. Due to its superior solubility (data not shown) in organic solvents, softisan® 100 was chosen as the model lipid in the present study. The concentration of softisan® 100 in the organic phase was 20 mg/ml; 3 ml of this solution was injected into 50 ml bidistilled water containing 0.1% polysorbate 80 (m/V). PCS data (Table 1) for all three studied solvents show monomodal distribution (distribution of number) with an acceptable PI range. For the chosen conditions, isopropanol seems to be the best solvent in terms of particle size and distribution as well as reproducibility and reliability. The z-average is about 150 nm with a PI of about 0.200. However, ethanol and acetone are also suitable. LNP produced with ethanol as solvent have a slightly larger diameter and PI (about 160 nm and 0.280, respectively) than LNP produced with isopropanol. The use of acetone as organic solvent causes significantly larger nanoparticles with a z-average of more than 200 nm and a PI of around 0.210. With regard to yield (see Table 2) as the parameter for the efficiency of LNP formation, acetone is the best solvent, providing a yield of over 95%. Ethanol and isopropanol with yields of 88.6% and 90.0%, respectively, are also efficient solvents for LNP preparation. An explanation for this observation may be the higher solubility of softisan® 100 in acetone and therefore a delayed precipitation of softisan® 100 from the organic phase allowing a longer diffusion between aqueous and solvent phases and resulting in a more effective and complete precipitation of the lipid in the form of LNP.

Table 2
LNP yield in different solvents ($n = 9$)

Solvent	Yield ± SD (%)
Acetone	96.5 ± 2.0
Ethanol	88.6 ± 3.4
Isopropanol	90.0 ± 1.5

3.3. Effects of process parameters

In the next step the influence of several process parameters on particle size and distribution was evaluated. Due to the results from the reproducibility assessment isopropanol was chosen as the model solvent. In general, if not otherwise mentioned, 1 ml of isopropanol with a softisan® 100 concentration of 20 mg/ml was injected into 30 ml of bidistilled water without emulsifier in order to keep process parameters to a minimum.

Firstly, the influence of the injected amount was investigated. An increase of the injected volume of solvent (Fig. 1a) with a constant lipid concentration (20 mg/ml) initially causes a slight decrease of the particle size and distribution. LNP size and distribution remain almost constant up to the addition of 10 ml of solvent–lipid solution. The addition of 10 ml of solvent results in larger LNP with a coarser particle size distribution. These findings suggest that the injected amount of solvent is of minor influence with respect to the particle size and distribution as long as a critical amount is not exceeded. Accordingly, it is important during the preparation process to avoid exceeding the critical solvent/water ratio (being between 1:6 and 1:3 for the examined systems). Fig. 1b shows the influence of an increase in the lipid concentration in the solvent phase at constant injected volume (1 ml). An increase of the lipid concentration leads to a concentration-dependent increase in particle size. Yet, the particle size distribution remains practically unchanged. A possible explanation for this observation may be the reduction of the diffusion rate of the solute molecules

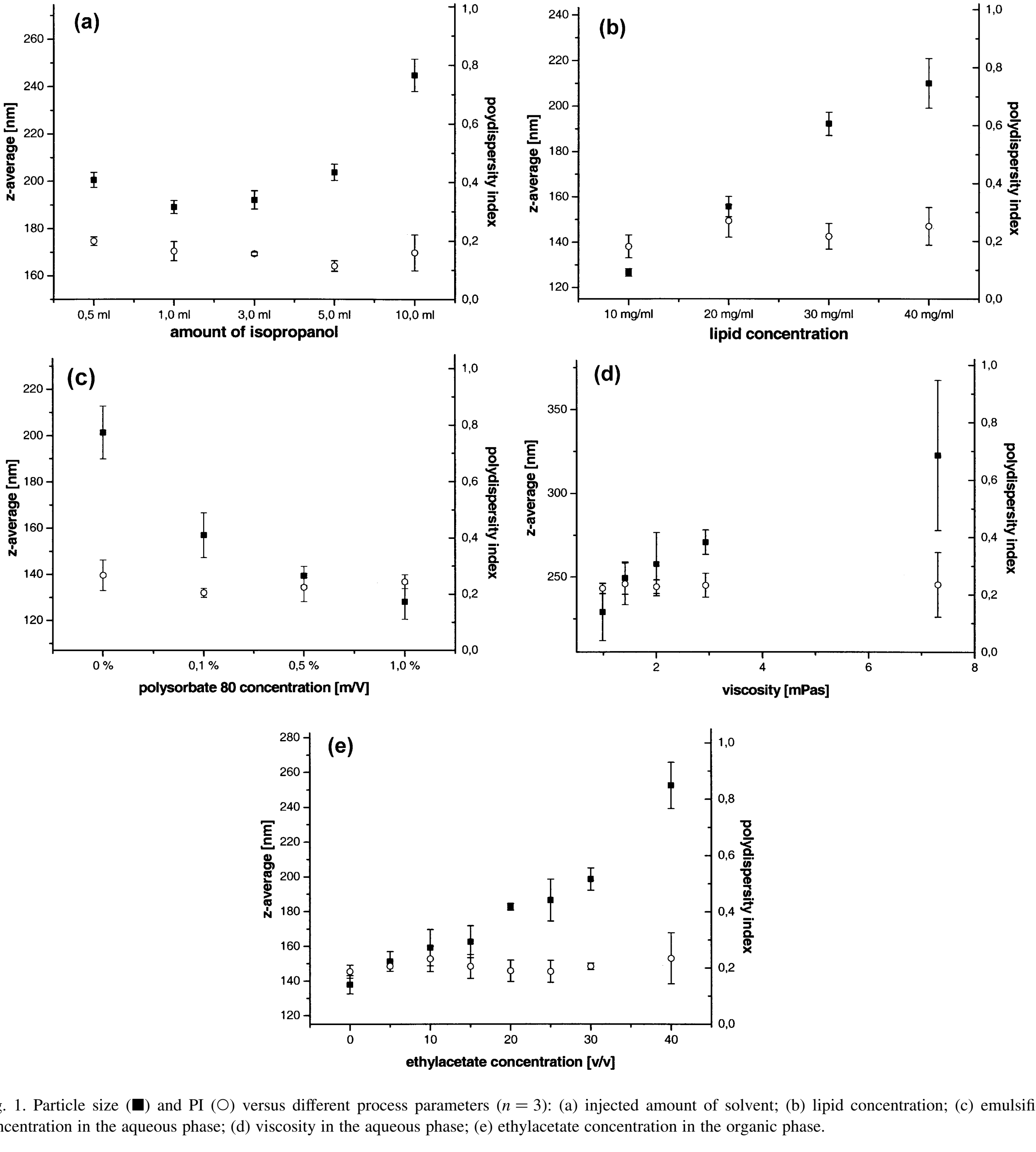

Fig. 1. Particle size (■) and PI (○) versus different process parameters ($n = 3$): (a) injected amount of solvent; (b) lipid concentration; (c) emulsifier concentration in the aqueous phase; (d) viscosity in the aqueous phase; (e) ethylacetate concentration in the organic phase.

in the outer phase as a result of the viscosity increase in the lipid–solvent phase with rising lipid concentrations. In Fig. 1c the influence of polysorbate 80 as model o/w emulsifier on LNP size and distribution is demonstrated. Already at the lowest emulsifier concentration of 0.1% a distinct particle size decrease from more than 200 nm with no emulsifier to about 155 nm is observed. Further addition of polysorbate 80 leads to a further decrease of the particle size down to 130 nm, though the particle size distribution remains more or less unchanged. With regard to particle size the emulsifier concentration seems to be an important process parameter. The emulsifier-induced decrease of the surface tension between organic and aqueous phases seems to allow the formation of initially smaller solvent droplets at the site of solvent injection causing the observed particle size decrease. In addition, the emulsifier helps to stabilize the newly generated surfaces and to prevent particle aggregation.

The LNP particle size is also influenced by the viscosity of the outer phase. As Fig. 1d illustrates, an increase in

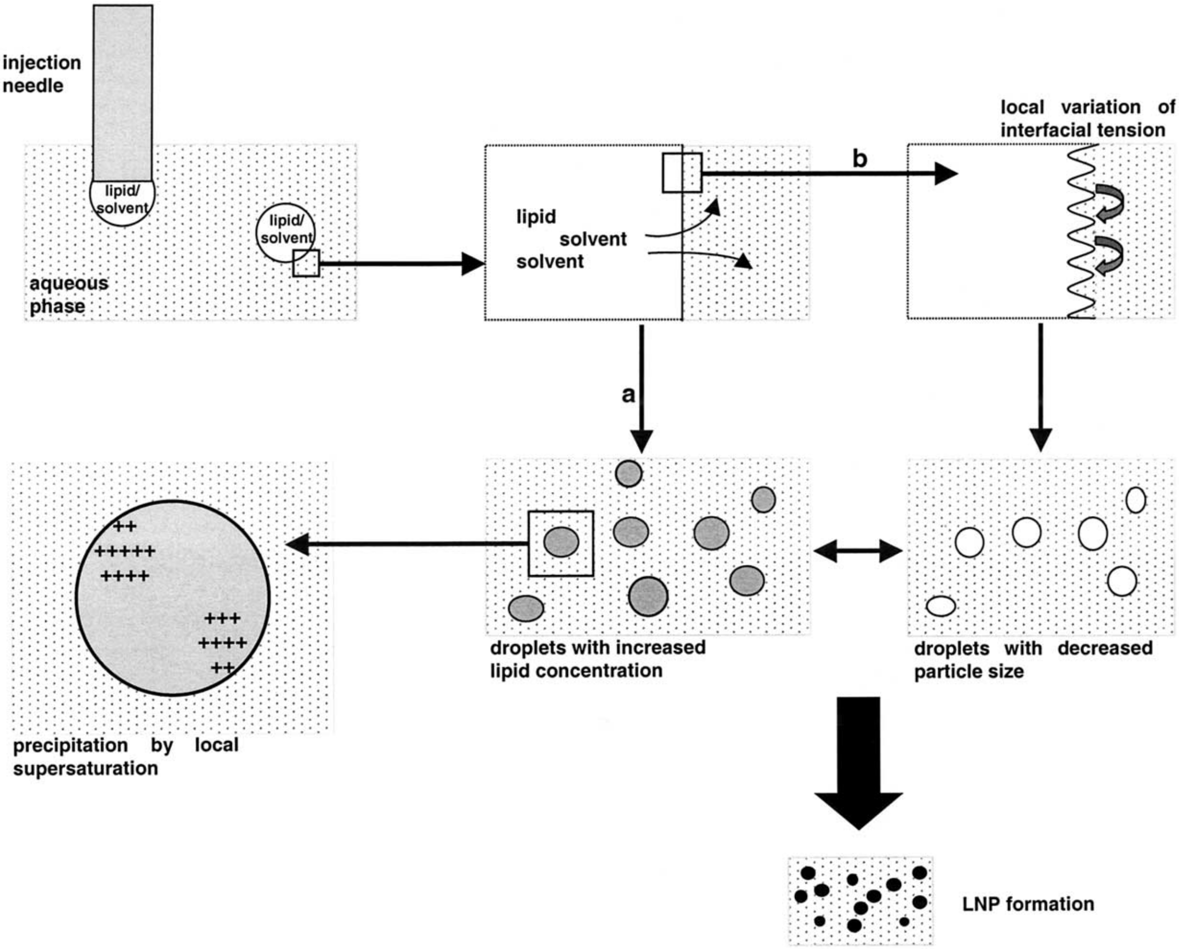

Fig. 2. Schematic representation of LNP formation.

viscosity of the aqueous phase on addition of glycerol leads to a concentration-dependent increase of the particle size without a major influence on the particle size distribution. This result suggests that a reduced diffusion rate of the solute molecules caused by an increased viscosity of the outer phase may be responsible for the particle size shift. During the screening of the 'solvent injection' method we noticed that it was impossible to produce LNP with ethylacetate alone as the organic solvent. For this reason, isopropanol was gradually blended with ethylacetate, and particle size and particle size distribution were examined. Fig. 1e shows that the particle size rises gradually with the ethylacetate concentration without a major influence on the particle size distribution. At ethylacetate concentrations beyond 40% no LNP could be obtained resulting in a *z*-average of more than 900 nm and a PI of around 1. As Fessi et al. [10] outline, a prerequisite for the solvent diffusion technique is a miscibility between solvent and aqueous phases in all ratios. By adding ethylacetate stepwise into the organic solvent the diffusion coefficient is changed – probably reduced (no data for infinite dilution of isopropanol in water are available yet) – but more importantly the solvent is transformed into only a partially water-miscible solvent. Hence, LNP formation is no longer possible beyond a critical ethylacetate concentration. This result supports data from Section 3.2 which has already indicated that the solvent is one important parameter which seems to control the particle size of the LNP. In addition, the particle size can be exactly adjusted by blending different solvents.

Taking all these observations into account we tried to develop a model for the LNP formation process (Fig. 2). The solvent injection technique seems to rely on the rapid diffusion of the solvent across the solvent–lipid interface with the aqueous phase [11]. Thus, the diffusion rate of the organic solvent through the interface seems to be a critical parameter for particle size determination. This hypothesis is in accordance with the results represented in Fig. 1d. Gradual solvent diffusion (see Fig. 2a) out of the in situ formed droplets into the continuous phase leads to a reduction of the droplet size and simultaneously to an increase of the lipid concentration within the droplets. Local supersaturated regions are formed and due to the instability of these supersaturated lipid–solvent solutions precipitation of the lipid takes place. The presence of an emulsifier within the aqueous phase helps to stabilize these supersaturated regions and the LNP until solvent diffu-

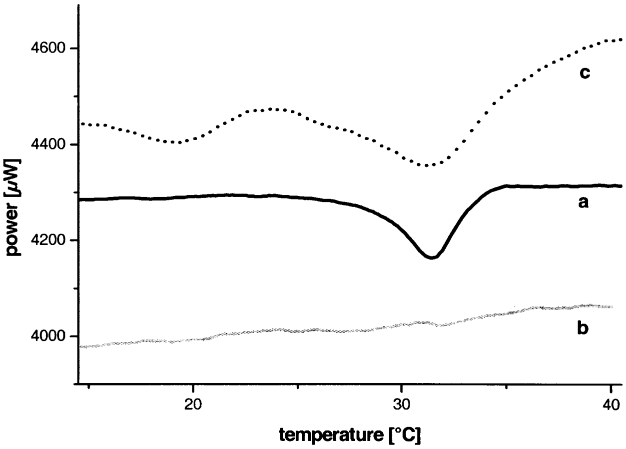

Fig. 3. DSC thermogram of softisan® 100: (a) bulk (solid black line); (b) LNP directly after preparation (solid grey line); (c) LNP after 4 weeks of storage (dotted black line).

sion is complete. Furthermore, the stabilizing agent causes a reduction of the surface tension between water and solvent resulting in smaller solvent–lipid droplets at the site of injection. The decrease in the particle size shown in Fig. 1c confirms this assumption. According to Davies and Rideal [12], the diffusion of the pure solvent from the lipid–solvent droplet provokes local variations in the interfacial tension of the droplet surface (see Fig. 2b). These variations decrease the interfacial tension and induce interfacial turbulence and pulsation between the two liquid phases. The interfacial turbulence and pulsation causes a breakdown of the originally formed droplet into smaller droplets with only a minor change of the lipid concentration within the droplet. The energy required for this process being described as ‘diffusion and stranding’ is provided by the free energy which is released as the solvent is redistributed to its equilibrium state. In our opinion both these effects which take place simultaneously and cannot be separated from each other contribute to the very effective formation of LNP by the solvent injection technique.

3.4. Physicochemical characterization

X-Ray diffraction studies of softisan® 100 show interference maxima of 4.20 and 0.38 nm both in Debye-Scherrer and goniometer analysis. Therefore, the existence of a β′-modification can be assigned to these interferences [13]. Thermoanalysis of the bulk (Fig. 3a) shows a single endothermic peak upon heating with a minimum of 31.4 °C and an enthalpy of 156.8 mJ/mg. The heat of fusion is in agreement with values given by Thoma and Serno [14] for a β′-modification of complex triglyceride mixtures. TEM analysis shows the typical layered structures of triglycerides (Fig. 4a).

In contrast, LNP of softisan® 100 display only diffuse halos on Debye-Scherrer X-ray diffraction analysis being at about the same region as the bulk. A possible explanation for this effect may be a reduced crystallinity of the LNP as well as the colloidal particle size of the LNP offering an insufficient number of diffraction levels. DSC thermograms of LNP measured directly after preparation (Fig. 3b) do not reveal any thermal event. After 4 weeks of storage two endothermic transitions with minima at 19.1 and 31.2 °C (Fig. 3c) can be detected. The latter transition corresponds to the melting of the β′-polymorph of the bulk. However, the enthalpy of this process cannot be determined due to the unsteady baseline. In addition, at about 19 °C a less

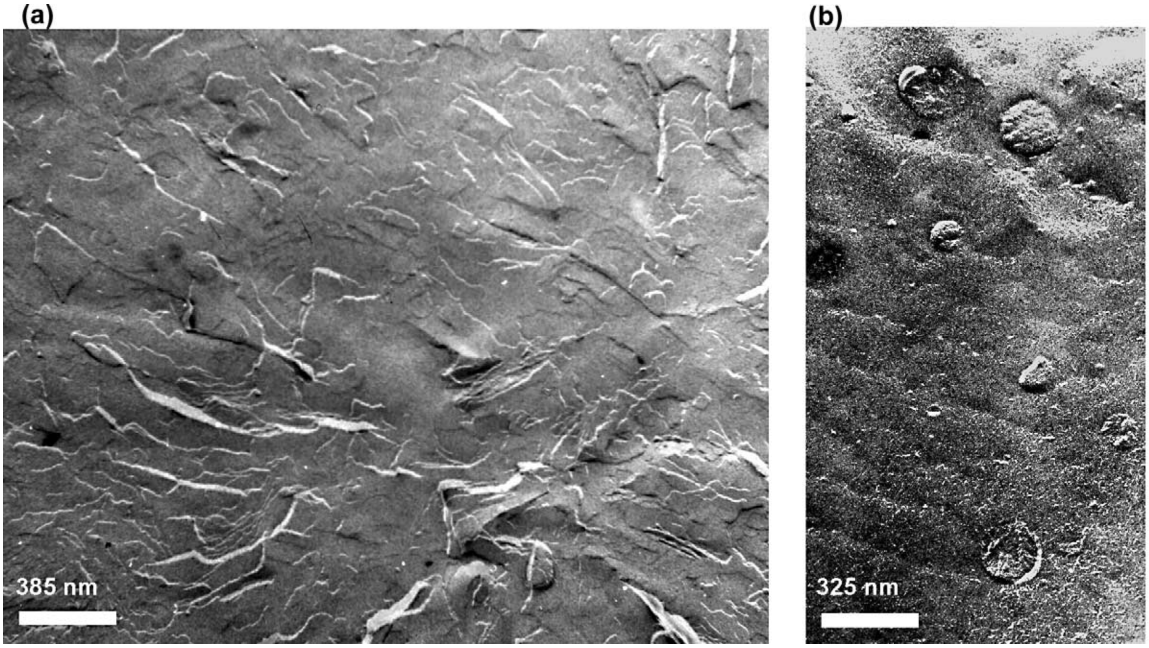

Fig. 4. TEM micrograph of softisan® 100: (a) bulk (bar corresponds to 385 nm); (b) LNP prepared with acetone as organic solvent (bar corresponds to 325 nm).

pronounced endothermic transition can be recognized; a possible explanation for this thermal event may be the coexistence of lipid being present in the α modification [8,15]. Due to the delayed recrystallization process of the colloidal lipid and the existence of at least two colloidal lipid modifications, a certain physical instability of the LNP has to be considered. TEM examination of the LNP (Fig. 4b) revealed the existence of well defined slightly anisometrical particles with homogeneous size and surface. The particle size of LNP prepared with acetone is between 90 and 320 nm which is in accordance with prior PCS measurements. The surface of the particles displays almost no layered structures which are similar to the bulk. Summarizing TEM, X-ray diffraction and DSC analysis, a distinct decrease in the crystallinity of the colloidal lipid in comparison to the bulk lipid is observed. These differences have been attributed to the high surface to volume ratio of glyceride nanocrystals by Siekmann and Westesen [16].

4. Conclusion

This study demonstrates that 'solvent injection' represents a viable new alternative for preparing LNP. The technique is efficient, versatile and of simple implementation. The particle size can be influenced and controlled by variation of process parameters such as injected solvent, lipid concentrations, injected amount and viscosity. One crucial parameter for the nanoparticle formation process seems to be the diffusion of the solvent from the lipid–solvent phase into the aqueous phase, although the entire process is not fully understood and further investigations are necessary. The degree of crystallinity of the colloidal lipid is distinctly reduced in comparison to the bulk lipid. A disadvantage of the 'solvent injection' method is the use of organic solvents. Although the solvents are pharmaceutically acceptable with regard to the patient they have to be considered very carefully since some promising routes of administration are excluded a priori and solvents may even harm the patient. For that reason, a complete removal of the solvents by evaporation, freeze-drying, ultrafiltration and washing of the LNP, etc. would be promising and worth studying.

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