Characterization of lipid nanoparticles by differential scanning calorimetry, X-ray and neutron scattering

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Abstract

Differential scanning calorimetry and X-ray diffraction play a prominent role in the characterization of lipid nanoparticle (LNP) dispersions. This review shortly outlines the measurement principles of these two techniques and summarizes their applications in the field of nanodispersions of solid lipids. These methods are particularly useful for the characterization of the matrix state, polymorphism and phase behavior of the nanoparticles which may be affected by, for example, the small particle size and the composition of the dispersions. The basics of small angle X-ray and neutron scattering which are also very promising methods for the characterization of LNPs are explained in some more detail. Examples for their use in the area of solid LNPs regarding the evaluation of particle size effects and the formation of superstructures in the nanoparticle dispersions are given. Some technical questions concerning the use of the different characterization techniques in the field of LNP research are also addressed.

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Contents

1. Introduction .............................................................. 380

2. X-ray and neutron scattering ..................................................... 380

2.1. Some considerations on the methods ............................................. 380

2.2. Aspects of basic scattering theory important for scattering experiments on LNPs ...................... 381

2.2.1. Scattering of a single electron ............................................ 381

2.2.2. Scattering of an ensemble of electrons........................................ 382

2.2.3. Scattering of a volume element with homogeneous electron density — scattering from the dispersion medium . . 382

2.2.4. Scattering of a dispersion of lipid nanocrystals ................................... 382

2.3. What can be learned from the complex scattering functions of LNP dispersions?...................... 384

2.3.1. Determination of particle size distributions on a molecular scale . . 384

2.3.2. How to interpret shifts of SAXS peaks in LNP dispersions? ............... 384

2.3.3. Scattering of medium and highly concentrated LNP dispersions .......... 386

2.4. Wide angle X-ray diffraction ................................................. 386

2.5. Some instrumental aspects of small angle scattering ..................... 386

3. Differential scanning calorimetry (DSC) ............................................... 388

4. Applications of DSC and X-ray diffraction for the characterization of LNP dispersions ............... 390

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1. Introduction

As for all drug delivery systems detailed characterization is a major part of the research and development work on lipid nanoparticle (LNP) dispersions to ensure the generation of systems with the desired properties. Among the multitude of analytical techniques employed for that purpose, differential scanning calorimetry (DSC) and X-ray diffraction (XRD) play a prominent role because they are able to provide structural information on the dispersed particles. Moreover, the use of these two techniques often leads to complementary information on the systems of interest. The background of these methods will be outlined in this review in a relatively brief way since DSC and XRD are well-known standard techniques in the area of pharmaceutics and since data evaluation from these methods is usually straightforward. In addition to XRD, the related techniques of small angle X-ray and neutron scattering (SAXS and SANS, respectively) can give very interesting additional information on the structure of the systems. Because these techniques are less frequently employed for the study of lipid nanoparticle dispersions, a more detailed introduction into their theoretical basics and their measurement principles will be given with special regard to their application in lipid nanoparticle research. In particular, we will consider the specific technical challenges arising from the investigation of LNP dispersions with these methods.

The second part of the review focuses on the characterization of specific properties of lipid nanoparticles that can be studied with DSC and XRD and also demonstrates the advantages of combining the two techniques. Moreover, we would like to focus the attention of the reader on some peculiarities of the use of DSC and XRD on LNP dispersions as the investigation of nanoparticulate material in liquid dispersion is not a common part of the technical literature.

This article will only cover investigations on nanodispersions based on solid lipids (in some cases with the admixture of liquid ones) which may, however, form solid, liquid as well as liquid crystalline particles in the dispersed state. Moreover, to limit the scope of our contribution, we will only consider matrix-type LNPs (e.g., emulsion droplets, suspension particles) although the analytical techniques under discussion here have important applications for the characterization also of membrane-type lipid particles (e.g., liposomes, dispersions of bicontinuous cubic phases) [1–5].

It should be emphasized that nanoparticulate lipids are not only of high interest as drug carrier systems but also play an important role in other fields of science. As a prominent example, dispersions of glycerides, in particular of triglycerides, are important research subjects in food technology (e.g., with respect to the behavior of milk and cream). Therefore, much basic work on the behavior of such systems originates from that area, providing a valuable resource on basic questions in this field. Often DSC and XRD are employed as major analytical techniques in these studies [6–10]. Another example is investigations on biological systems such as lipoproteins for which also a wealth of information has been collected by the techniques presented here [11–14].

2. X-ray and neutron scattering

2.1. Some considerations on the methods

The properties of formulations of LNPs are determined mainly by the manifold structures formed by the different components of the dispersions (lipid, drug, stabilizers, dispersion medium) and their interactions. Characteristic lengths $d$ of these structures range from sub-atomic distances ($d < 1$ Å) important for e.g. crystal structure determination up to the μm range corresponding to the size of large particles or particle assemblies. The whole size range can be investigated by means of X-ray and neutron scattering. In general those methods detect electron (X-ray scattering) and nucleus (neutron scattering) density fluctuations, respectively, on a length scale $d$ according to Bragg's law

$$2d \sin \theta = \lambda. \quad (1)$$

Considering the experimentally available wavelength range $0.5 \, \text{Å} < \lambda < 20$ Å for neutrons and $0.5 \, \text{Å} < \lambda < 2.5$ Å for X-rays the scattering angles $2\theta$ corresponding to the size range of interest are between $0.01^\circ$ and $180^\circ$. Several other properties of X-rays and neutrons such as their non-destructive nature with respect to the sample structure, their high penetration capability for organic systems allowing bulk property determination, their sensitivity to small structural changes and the variety of their applications which will be highlighted in the following give the reason for the use of these probes in LNP research.
Corresponding to experimental equipment two different techniques for elastic scattering are to be distinguished:

1. X-ray (SAXS) and neutron (SANS) small angle scattering and
2. X-ray (XRD) and neutron (ND) diffraction, also called wide angle scattering.

The only difference between both method types is the range of scattering angles $2\theta$. While standard diffractometers cover angles between about $5^\circ$ and $180^\circ$, the range between $0.01^\circ$ and $3^\circ$ is typical for small angle instruments.

Most popular applications are the identification of crystal structures, modifications, particle sizes and shapes as well as quantitative phase analysis (quantitative determination of the crystalline components in a formulation) and determination of crystallinity indices. The interpretation of diffraction data is often straightforward, e.g. for the determination of the modification of triglyceride nanocrystals. In contrast, it may be very complicated e.g. to get reliable information on a particle size distribution of highly concentrated nanodispersions from SAXS or SANS data. For many investigations with X-ray and neutron scattering there is more information about the sample in the data than usually extracted by the scientist. On the other hand some artifacts due to a special instrumentation can lead to misinterpretation as e.g. seemingly vanishing Bragg intensities for strongly textured samples or arbitrary multiple intensity peaks for poor powder samples in a Kratky camera (cf. Section 2.5). In order to make it easy to get reliable information on a particle size distribution of highly concentrated nanodispersions from SAXS or SANS data.

For many investigations with X-ray and neutron scattering there is not change its energy $E$ during the scattering process$^1$: $|\mathbf{k}_i|=|\mathbf{k}_f|$ (cf. Fig. 1). For this case the general equation (derived from Fig. 1 using cosine theorem) $Q^2 = \mathbf{k}_i^2 + \mathbf{k}_f^2 - 2\mathbf{k}_i \cdot \mathbf{k}_f + 4 h^2 \mathbf{k}_i \mathbf{k}_f \sin^2 \theta$ reduces to

$$Q = \frac{2\pi}{d} = 2\pi s = \frac{4\pi \sin \theta}{\lambda}$$

with $Q = |\mathbf{Q}|$ and $s=1/d$. $s$ is often used in the literature on small angle scattering instead of $Q$. It should also be mentioned here that from $E = mc^2 - E/hc(\lambda)$ and $p = mc - h/\lambda$ it is straightforward that $\mathbf{p} = h\mathbf{Q}$ represents the momentum transferred from the sample to the X-ray. The same term holds for material waves as neutrons according to predictions of de Broglie [25].

By means of some general considerations about X-ray scattering by electrons some basic results from scattering theory which are also valid for neutron scattering and relevant for investigations of LNPs will be derived phenomenologically in the next sections. Some special effects observed for LNPs are included. A general expression for the calculation of the scattering function of nanocrystalline dispersions will be derived and discussed.

2.2. Aspects of basic scattering theory important for scattering experiments on LNPs

The validity of Bragg’s law (Eq. (1)) is limited to the case of elastic scattering which means that the X-ray or neutron does not change its energy $E$ during the scattering process$^1$: $|\mathbf{k}_i|=|\mathbf{k}_f|$ (cf. Fig. 1). For this case the general equation (derived from Fig. 1 using cosine theorem) $Q^2 = \mathbf{k}_i^2 + \mathbf{k}_f^2 - 2\mathbf{k}_i \cdot \mathbf{k}_f + 4 h^2 \mathbf{k}_i \mathbf{k}_f \sin^2 \theta$ reduces to

$$Q = \frac{2\pi}{d} = 2\pi s = \frac{4\pi \sin \theta}{\lambda}$$

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By means of some general considerations about X-ray scattering by electrons some basic results from scattering theory which are also valid for neutron scattering and relevant for investigations of LNPs will be derived phenomenologically in the next sections. Some special effects observed for LNPs are included. A general expression for the calculation of the scattering function of nanocrystalline dispersions will be derived and discussed.

2.2.1. Scattering of a single electron

The kinematic scattering theory [15], which assumes that the incident and scattered waves are not influenced by the sample, is an adequate approximation for weakly scattering samples, as normally used in experiments with LNPs. Thus extinction, refraction and absorption are not considered in the following but can be introduced separately [15]. The same holds for the influence of thermal motions of the atoms in the sample on the X-ray interferences (Debye–Waller factor) and corrections specific for special experimental setups such as polarization and Lorentz factors. We consider an incident X-ray wave (cf. Fig. 1) of the form $E_0^x = E_0 e^{i\omega t - k_0 x - k_0 r}$. The wave propagates in $x$ direction and is polarized in $z$ direction as indicated by the superscript $z$. For this geometry the electric field vector $E_0^x$ at point $R$ (cf. Fig. 2) of a wave scattered from a single electron

---

$^1 E=hc/\lambda$ for X-rays, $E=mc^2$ for neutrons with $h=2\pi k$: Planck’s constant, $c$: velocity of light in vacuum, $m_n$: rest mass of neutron.
positioned in the coordinate system’s point of origin results from Maxwell’s theory and is given by
\[
E_{0}^{\infty} = \frac{e^{2} \sin \theta_{z}}{m_{e} c^{2} R} E_{0} e^{i \omega t - \mathbf{k} \cdot \mathbf{r}}
\]
(3)
with \(e\): elementary charge and \(\theta_{z}\): angle between \(z\) and \(z'\). This wave propagates along \(z'\) and is polarized in \(z'\) direction. The scattering amplitude \(E_{0}^{\infty}\) of an electron positioned at \(\mathbf{r}_{e}\) is according to Fig. 2 and using Fraunhofer’s approximation \(X_{1} + X_{2} \approx Y_{e} R + Y_{e} (\mathbf{q}^{-3} - 3)\) given by
\[
E_{fr_{e}}^{\infty} = \frac{e^{2} \sin \theta_{z}}{m_{e} c^{2} R} E_{0} e^{i \omega t - \mathbf{k} \cdot \mathbf{r}}
\]
(4)
The intensity scattered from a single electron can be calculated by multiplication of \(E_{fr_{e}}^{\infty}\) with its complex conjugate:
\[
I_{e} = \frac{e^{4} \sin^{2} \theta_{z}}{m_{e}^{2} c^{4} R^{2}} |E_{0}^{\infty}|^{2}
\]
(5)

2.2.2. Scattering of an ensemble of electrons
The intensity scattered from \(n\) electrons can accordingly be calculated after adding up Eq. (4) for all \(n\) electrons:
\[
I_{n}(\mathbf{Q}) = I_{e}(\mathbf{Q}) \cdot \sum_{k=1}^{n} \sum_{j=1}^{n} \cos \left( \mathbf{r}_{e_{j}} - \mathbf{r}_{e_{k}} \cdot \mathbf{Q} \right)
\]
(6)
An important interpretation of this equation is that the total scattered intensity of an ensemble of \(n\) electrons is proportional to \(n^{2}\) if the electrons are within a volume with dimensions small compared to the X-ray’s wavelength and therefore \((\mathbf{r}_{e_{j}} - \mathbf{r}_{e_{k}}) \cdot \mathbf{Q} \ll 1\) if \(j \neq k\). This is because for \(j \neq k\) the argument of the cosine is equally distributed over the whole codomain during the summation leading to a mean contribution to the sum of zero. The remaining terms for \(j = k\) lead to the mentioned proportionality.

From these considerations we can now understand the result from small angle scattering theory that for widely separated particles the scattering intensity is proportional to the square of the electron density of a single particle but only proportional to the number of particles [18]. The following consequence for the total scattering of an LNP dispersion might be surprising at a first view: We compare the total scattering intensities of two low concentrated LNP dispersions both with the same total mass of particles that means with the same total number of electrons inside the particles. The first dispersion has half the number of particles but with double weight of each compared to the second dispersion. Whereas the total mass of the scattering particles is the same for both dispersions the total scattering of the first dispersion has twice the intensity of the one of the second dispersion.

2.2.3. Scattering of a volume element with homogeneous electron density — scattering from the dispersion medium
Assuming a large sample with a homogeneous electron density the same argument introduced above is valid with regard to the calculation of the scattered intensity to a fixed \(\mathbf{Q}\). For small distances \(l_{jk} = |\mathbf{r}_{e_{j}} - \mathbf{r}_{e_{k}}|\) of infinitesimal volume elements with the constant electron density \(\rho_{e}\) all cosine values are positive. But for increasing \(l_{jk}\) the cosine values will change alternately their signs. In this case the total scattered intensity will be essentially zero except for extremely small angles. If the sample is large enough these angles are experimentally not accessible. According to Babinet’s theorem it can be followed that the scattering intensity \(I(\mathbf{Q})\) of a small particle with homogeneous \(\rho_{e}\) cannot be distinguished from the scattering of an infinite large sample with the same electron density but with a cut-out of the size of the small particle. By means of this theorem the amplitude \(E_{0} \cdot S_{D_{k}}^{0}\) of the X-ray scattered by the dispersion medium surrounding a crystal \(k\) of parallelepiped shape (cf. Section 2.2.4) can be calculated:
\[
S_{D_{k}}^{0} = \frac{V^{k} \rho_{D}}{N_{k}^{1} N_{k}^{2} N_{k}^{3}} \prod_{j=1}^{3} \frac{1}{(k_{j} - k_{j})} \alpha_{j} \left( e^{[(k_{j} - k_{j}) \mathbf{a}_{j}^{0}]} - 1 \right)
\]
(7)
\((\mathbf{a}_{j}^{0})\): fundamental lattice vectors of crystal \(k\), \(N_{k}^{j}\): number of unit cells of crystal \(k\) in \(\mathbf{a}_{j}^{0}\) direction, \(V^{k}\): total volume of crystal, \(\rho_{D}\): mean electron density of dispersion medium).

Another important conclusion of the discussion above is that the scattering of a particle with an electron density \(\rho_{e}\) cannot be observed in a dispersion medium with the same electron density. This holds only for homogeneous electron densities. In real particles or dispersion media the electron densities vary in space on a certain length scale \(l\). But if we talk about \(Q\)-values small with respect to \(1/l\) these fluctuations cannot be observed. Therefore the scattering intensity of e.g. an LNP dispersion is proportional to the difference of the averaged electron densities of the particles and the dispersion medium (called the scattering contrast). This is correspondingly true for neutron scattering, where the scattering contrast is easier to change. This holds especially for aqueous suspensions where water can be partly or fully exchanged by \(D_{2}O\). The coherent scattering cross section of deuteron is three times larger than that of hydrogen [26]. Hence, for typical LNP dispersions the scattering contrast between the particles and the dispersion medium is for neutrons (when using \(D_{2}O\)) much larger compared to that of X-rays. This compensates the much lower primary beam intensities at neutron instruments compared to X-ray beam lines to a large extent and measuring times of only some minutes per diffraction pattern are required for good statistics.

2.2.4. Scattering of a dispersion of lipid nanocrystals
Crystalline nanoparticles of triglycerides in their stable \(\beta\)-modification exhibit the shape of a parallelepiped with one dimension much smaller compared to the others (Fig. 17 bottom).
The alkyl chains of the triglyceride molecules (long c-axis of the unit cell) are oriented along that small dimension, which is referred to as thickness of the crystal. A simplified (2D) schematic representation of such a nanocrystal (with a thickness equal to that of two unit cell heights) is displayed in Fig. 3. On the basis of crystals of parallelepiped shape it is also possible to approximate the shape of other crystals. Therefore this model seems to be well suited to be used for simulation calculations of X-ray patterns of nanocrystalline dispersions and will be described in this section.

The amplitude of the electric field of the scattered wave from a volume element of a dispersion of nanocrystals, which is small compared to the coherence volume of the X-rays, can be calculated using the vectors \( \vec{r}_i \) defined in Fig. 3 by

\[
E_{\text{tot}}^{z'}(Q) = \sum_{k=1}^{N_k} \sum_{m_1=0}^{N_1-1} \sum_{m_2} \sum_{m_3=0}^{N_3-1} \int dV \sum_{j=1}^{N_j} \rho_j \rho_{j',d} dV.
\]  

The scattering contribution of each of the \( N_j \) atoms in the unit cell is calculated by the integration of the amplitudes \( E_{\text{tot}}^{z'} \) scattered by the electron density \( \rho_j \). The integration variable is the vector \( \vec{R} \) (cf. Fig. 3) and the integration is performed over volume \( V_\lambda \) of the \( j \)th atom in the unit cell. This integration does not need to be performed for simulation calculations but the results are tabulated as atomic form factors in the International Tables for X-ray Crystallography [28]. After the calculation of the scattering contribution of all atoms of a single unit cell we have to sum up the contributions of all unit cells in the crystal designated by the vectors \( m_1a_i + m_2b_j + m_3c_k \) with \( m_3 \in [0,N_3^{k}-1] \). The last sum adds the scattering contributions of all \( N_k \) nanocrystals in the respected sample volume referenced by \( \vec{R}_0 \).

It can be stated here that the coherence volume for X-ray as well as for neutron diffraction experiments exceeds the size of particles and particle associates of interest in LNP research, which means that the diffraction patterns should include all relevant structural information.3

For a dispersion of crystals of parallelepiped shape \( E_{\text{tot}}^{z'}(Q') \) can be written as:

\[
E_{\text{tot}}^{z'}(Q) = E_{f}^{0} \sum_{k=1}^{N_k} e^{i \vec{R}_k \cdot \vec{Q}} \left( F_k - G_k - S_{D_k} \right)
\]

with the structure amplitude \( F_k \) comprising the scattering of the atoms in the unit cell of the \( k \)th crystal, the lattice amplitude \( G_k \) describing the interference of the periodically arranged unit cells and \( S_{D_k} \) being an approximated term which summarizes the scattering of a quasi continuous dispersion medium. It is obvious that \( F_k \), \( G_k \) and \( S_{D_k} \) are functions of \( Q \). \( F_k \) and \( S_{D_k} \) vary smoothly with \( Q \), whereas \( G_k \) exhibits distinct sharp maxima around \( Q = G \) which are responsible for the Bragg reflections in \( I(\vec{Q}) \). \( G \) represents any linear combination of the fundamental vectors of the reciprocal lattice [20].

The product of \( E_{\text{tot}}^{z'} \) with its complex conjugate gives the intensity of the scattered X-rays:

\[
I(\vec{Q}) = (\Delta \lambda)^2 \sum_{\lambda} \left( E_{\text{tot}}^{z'}(\vec{Q}) \cdot (E_{\text{tot}}^{\dagger z'}(\vec{Q}))^\star \right).
\]  

From this multiplication cross-terms arise besides the \( |F_k|^2 \) and \( |G_k|^2 \) terms. For a single particle there is only one cross-term \( -2Re(\xi_k F_k G_k^\star) \) (\( Re(\lambda) \) designates the real part of \( \lambda \)). This cross-term is some kind of interference term between particle and solvent scattering, which influences the small angle scattering of LNP dispersions significantly as demonstrated in Section 2.3.2. For an ensemble of particles also interparticle terms arise, which describe the interference of the scattering of different particles. These terms influence and dominate in some cases the small angle scattering curves of medium and highly concentrated SLN dispersions as discussed in Section 2.3.3.

According to the limited size of the coherence volume the vector \( \vec{R}_0 \) (cf. Fig. 3) shall designate the point of origin of a coherence volume element. A crude approximation to calculate the scattering of the whole sample is to multiply \( I(\vec{Q}) \) with the total sample volume divided by the coherence volume.

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3 The transverse (spatial) coherence lengths of X-rays and neutrons with wavelength \( \lambda_0 \) in diffraction experiments can be estimated by \( \xi_{x,y} \approx l_{aperture} / l_{c} \) (\( l_\lambda \) distance from source to sample (collimation length), \( l_{c,z} \): lateral dimensions of the source in \( y \) and \( z \) direction, respectively). If the primary beam is collimated by apertures in a way that the full source size is not seen by the sample the lateral aperture dimensions and the distance from aperture to sample have to replace \( l_{aperture} \) and \( l_\lambda \), respectively. The longitudinal (temporal) coherence length \( \xi_\lambda \) can be estimated according to the approximated equation known from classical optics \( \xi = \frac{\lambda_0}{2 \Delta \lambda} \) for Bragg diffraction by \( \frac{\lambda}{2 \Delta \lambda} \) where \( \Delta \lambda \) is the full width at half maximum (FWHM) of the wavelength spectrum of the primary beam.
2.3. What can be learned from the complex scattering functions of LNP dispersions?

2.3.1. Determination of particle size distributions on a molecular scale

For small crystals of only a few unit cells thickness the Bragg peaks are significantly broadened due to the lattice amplitude, which is not yet sharply peaked. Therefore information on the particle thickness distribution of an LNP dispersion is contained in the width and shape of the Bragg reflections. From a single Bragg reflection at \( G = G \) the thickness distribution of the particles parallel to \( G \) can be deduced. The analysis of different reflections can be used to distinguish between particle thicknesses along the corresponding directions. If one or more higher orders of a reflection can be measured simultaneously strain and size effects which both contribute to Bragg peak broadening can be separated. For the case of triglyceride particles also a single line evaluation excludes the influence of strain effects [29].

Nanoparticles of \( \beta \)-triglycerides are platelet-like shaped with a diameter typically in the range between 50 nm and 300 nm and a thickness about a factor ten smaller. The size and aspect ratio of the particles are influenced by the emulsifiers, the type and modification of the triglyceride and the preparation conditions [30–32]. The large surfaces of the platelets are parallel to the crystallographic (001)-plane. Thus the thickness distribution of the platelets can be evaluated from the (001)-reflection which occurs in the small angle regime. It is obvious that a single diffraction peak does not contain enough information in order to extract a complex size distribution function. In the case of triglycerides it is, however, possible to collect many SAXS patterns over the broad melting temperature range of the particles (cf. Section 4.3). The diffractograms produced by subtraction of two SAXS patterns collected at only slightly different temperatures yield information on the particles melted in that temperature range. Such difference diffraction patterns but with quite large temperature steps are displayed in Fig. 4. It could be demonstrated that the melting temperature of triglyceride nanoparticles decreases with their decreasing thickness, and particle size distributions with molecular resolution could be determined [29,33].

2.3.2. How to interpret shifts of SAXS peaks in LNP dispersions?

As discussed in Section 2.2.4 the calculation of \( I (\tilde{Q}) \) even with the simplified term for \( E_{\text{el}}^2 (\tilde{Q}) \) of Eq. (9) gives a large number of cross-terms of reasonable intensity contributions. This is because for crystalline nanoparticles the size of their unit cell, the crystal’s dimensions and the interparticle distances in a dispersion are within the same order of magnitude. This also holds for liquid crystalline and to some extent even for non-crystalline particles.

The relevance of the considerations above shall be demonstrated again for nanocrystalline triglyceride dispersions. From the temperature resolved SAXS experiments discussed above the position of the Bragg reflection on the s-scale for each particle size fraction can also be determined. It can be seen from Fig. 4 that this position is shifted continuously to lower s-values for decreasing particle thicknesses. Using Bragg’s law the peak shift could misleadingly be interpreted as an increase of the (001)-interplanar spacing with decreasing thicknesses of the nanocrystals. This interpretation would be in contradiction to the observations that lattice constants of nanocrystals decrease with decreasing particle size due to the influence of the interfacial tension.

Therefore simulation calculations were performed on the basis of Eq. (9). In Fig. 5 calculated scattering curves of tripalmitin nanoparticles with thicknesses of 1, 2 and more molecular layers are displayed. The position of the (001)-Bragg reflection of polycrystalline tripalmitin (powder sample) is marked in each subfigure by a vertical line. For the calculations the crystallographic data of a large tripalmitin crystal were used. Although the (001)-interplanar distance was kept constant in the calculations the position of the calculated (001)-Bragg reflection is continuously shifted to lower s-values with decreasing particle thickness. The simulation results are quantitatively in good agreement with the experimental results as illustrated in Fig. 6 or in [33].

The reason for the peak shift is an interference of the lattice amplitude, which represents still a broad peak function on the s-scale due to the small number of unit cells along \( G_{001} \), the structure amplitude and the scattering amplitude from the dispersion medium including the stabilizer layer. It is remarkable that the simulation results cannot be reproduced without the phospholipid layer. In this case the peak shift is negligible and not consistent with the SAXS data. In contrast to

![Fig. 4. Difference diffraction patterns of a temperature resolved SAXS measurement of a tripalmitin nanosuspension. During the experiment the temperature of the sample was increased stepwise. The displayed curves result from subtraction of diffraction patterns recorded at temperatures listed in the legend. The calculated average thickness of the particles which melted in the small angle regime. It is obvious that a single diffraction peak does not contain enough information in order to extract a complex size distribution function. In the case of triglycerides it is, however, possible to collect many SAXS patterns over the broad melting temperature range of the particles (cf. Section 4.3). The diffractograms produced by subtraction of two SAXS patterns collected at only slightly different temperatures yield information on the particles melted in that temperature range. Such difference diffraction patterns but with quite large temperature steps are displayed in Fig. 4. It could be demonstrated that the melting temperature of triglyceride nanoparticles decreases with their decreasing thickness, and particle size distributions with molecular resolution could be determined [29,33].](image)
recent cryo transmission electron investigations [36] the phospholipid layer could now clearly be distinguished from the triglyceride by X-ray scattering. Furthermore it was found that the geometry as well as the electron density distribution of the phospholipid layer deviates significantly from the situation in phospholipid double layers [37]. The shift of the (001)-peak is strongly influenced by the electron density difference between the continuous phase and the nanoparticle. As illustrated by the dashed line in the second subfigure of Fig. 5 the small angle scattering of a particle in vacuum is considerably more intense compared to that of the same particle in a dispersion medium, due to the increased electron density contrast, and the shapes of the two scattering curves are completely different. In the case of tripalmitin the simulations predict a shift to higher $s$-values when the electron density of the dispersion medium exceeds the one of water. Such an investigation is hard to do for X-rays. But an analogous SANS experiment with deuterated fat should be possible.

Fig. 5. SAXS patterns calculated for single tripalmitin nanoparticles surrounded by a phospholipid monolayer in an aqueous solution for different particle thicknesses (number of unit cells parallel to crystallographic c-axis). The dimensions of the crystals parallel to $a$- and $b$-axes were 120 nm and 100 nm respectively. The structure factor was calculated from the crystal data given in [34]. The phospholipid layer was approximated by two layers around the crystal of homogeneous electron density each with $\rho=310$ electrons per nm$^3$ (hydrophobic tail part, thickness $l=0.8$ nm) and $\rho=350$ electrons per nm$^3$ (hydrophilic headgroup part, $l=1.3$ nm), respectively (approximated from DPPC double layer simulation results e.g. [35]). The scattering amplitudes of these layers and the aqueous solution ($\rho=333$ electrons per nm$^3$) were calculated according to Eq. (7). When using approximated electron densities from DPPC double layer simulation results (e.g. $\rho=228$ electrons per nm$^3$ (hydrophobic tail part, thickness $l=1.1$ nm) and $\rho=398$ electrons per nm$^3$ (hydrophilic headgroup part, $l=1.2$ nm)) [35]) the calculated SAXS patterns do not fit to the experimental data. Displayed is the scattering intensity (for clarity in different arbitrary units), averaged over all particle orientations, as calculated by numerical integration. The dashed line in the second subfigure represents the scattering of the same particle (including the phospholipid layer) used for the solid curve on the same intensity scale but excluding the scattering of the dispersion medium (particle in vacuum).

Fig. 6. Dependence of the (001)-spacing $d_{001}$ on the particle thickness $D$. The data (circles) have been extracted from SAXS patterns of a tripalmitin nanodispersion characterized in the caption of Fig. 4. The data analysis has been performed as described in [33]. The triangles correspond to the values deduced from the simulations presented in Fig. 5.

Fig. 7. SAXS patterns of a native (10 wt.%) and diluted tripalmitin nanosuspensions recorded at the X33 beam line of the EMBL at DESY, Hamburg, Germany. The curves are shifted along the intensity scale for better visualization. The features due to the formation of stacked lamellae are marked with arrows for the 10% curve. With increasing $Q$-values the intensity of these features decreases but in the regime of the broad tripalmitin (001)-reflection around $s=0.248$ nm$^{-1}$ they are again amplified by interference with the Bragg scattering. The repeat distance of the lamellae is about 33 nm for the 10% dispersion and 42 nm for the 7% dispersion. For the dispersions with only 3 wt.% of tripalmitin no stacked lamellae are observed.
With increasing particle thickness the (001)-reflection narrows due to the shape of the lattice amplitude, which becomes more and more dominant. For 20 unit cells along the c-axis a typical Bragg reflection can be observed. The significant difference between the scattering of a single unit cell and a single tripalmitin layer is due to the different small angle scattering of both particles when $\bar{Q}$ is perpendicular to $\bar{G}(001)$.

2.3.3. Scattering of medium and highly concentrated LNP dispersions

Eq. (9) includes the interference effects between different nanocrystals. These effects can be avoided by dilution of the investigated dispersions so that the particles are not associated as illustrated in Fig. 7. For many systems one is, however, interested in the native formulation. For triglyceride dispersions the formation of stacked lamellae and even liquid crystalline arrangements has been reported [38–40]. For the case of stacked lamellae additional features in the $Q$-range below the (001)-reflection appear (cf. Fig. 7). A strong interference of higher orders of these features with the (001)-reflection leads to its partial (Fig. 7) or complete deformation as observed for DNA loaded tripalmitin nanodispersions [41]. In the latter case the stacked lamellae can be regarded as aggregates and thus the small angle features can also be observed at low particle concentrations. From the additional features the interparticle distances in the stacked lamellae and their relative frequency can be deduced. It should, however, be mentioned here that due to the complex interference of many cross-terms the interference maxima are not equidistant [38] as expected for a lamellar phase, which complicates a detailed analysis.

2.4. Wide angle X-ray diffraction

Wide angle X-ray diffraction is well-known in LNP research and can be regarded as an established method. Therefore it will be only briefly introduced here. Most X-ray studies that have been published for lipid nanoparticles are wide angle diffraction investigations. This is because much important information can be revealed from the crystalline state of drugs and excipients of an LNP formulation. First of all the crystalline ingredients of a dispersion could be analyzed quantitatively according to the characteristic diffraction patterns of the known structures (phase analysis). It should however be kept in mind, that the sensitivity of the method is low which means that an analysis of a component of less than 0.5 wt.% with respect to the total sample mass is difficult.

If a crystalline component is detected one gets also information on its crystal modification [42,43,31,44,45–47]. This is particularly important with respect to the matrix lipids which are very often polymorphic substances. Modification changes after preparation of a formulation are well-known to e.g. segregate the drug out of the nanocrystals and can thereby influence the drug release properties of the dispersions [48].

If the intensity of the used X-ray instrument is high enough even fast transitions of modifications can be resolved by subsequent detection of diffraction patterns in time [30,31,43,46,47]. An X-ray/DSC combination, which is very useful for LNP research, has been used at the beam line BL-15A of the source ‘Photon Factory’, Tsukuba, Japan [49] and at beam line 5.2 at ELETTRA synchrotron, Trieste, Italy [10]. Both complementary methods can be applied simultaneously on the same sample and thus a direct correlation of DSC signals and structural transitions is obtained.

2.5. Some instrumental aspects of small angle scattering

According to Eq. (1) it should be possible to overcome the problem of measuring at very small angles, which is experimentally intricate, when using longer wavelengths $\lambda$. The absorption of X-rays with wavelengths above 2 Å by the sample is, however, so high that the scattered beam is strongly attenuated and absorption corrections are difficult to apply [18]. For neutrons it is possible to use wavelengths up to more than 20 Å but the intensity of the available neutron beam decreases dramatically with decreasing neutron energy for all available neutron sources.

The basic principles of a small angle apparatus can be explained corresponding to the simple drawing in Fig. 8. First of all a radiation source for X-rays and neutrons, respectively, is needed. Conventional X-ray generators which produce X-rays by electron bombardment on a fixed or rotating anode target are used in commercially available laboratory instruments for both SAXS and XRD. The target material determines the resulting X-ray energy which is typically in the energy range between 5.4 keV ($\text{CrK}_\alpha$) and 24.9 keV ($\text{AgK}_\beta$). Besides the ‘Bremsstrahlung’ the spectrum of a copper target, which is used most often on pharmaceutical samples, is dominated by the two intense lines $\text{CuK}_\alpha$ at $\lambda=1.5405$ Å and $\text{CuK}_\beta$ at $\lambda=1.5443$ Å. The second line has about half the intensity of the first one and can be removed by a primary monochromator. If no primary

Fig. 8. Schematic representation of a simplified pinhole small angle scattering apparatus. The detector can be moved in the detector pipe to different distances from the sample as e.g. $l_1$ and $l_2$. The detector plane is perpendicular to the primary beam direction and is determined by its distance to the sample.
monochromator is used the CuKα line is usually reduced in intensity by a Ni-filter.

In the case of weakly scattering samples it is convenient to use more intense X-ray sources available at electron (or positron) synchrotrons. The electrons are accelerated up to several GeV and stored in large circular tubes. The X-rays are generated by deflection of the electrons by a magnetic field. Because of the relativistic velocity of the electrons the generated X-ray beam is extremely intense at an extraordinary brilliance $B = \frac{Nt}{\lambda \Omega}$ (N/t: number of emitted photons per time, $d\Omega$: solid angle, $dF$: area of source, $d\lambda/\lambda$: relative bandwidth). The use of special arrangements of periodic fields leads to even higher intensity and brilliance for polychromatic (wiggler) and monochromatic (undulator) beams. Measuring times for a small angle diffraction pattern at modern synchrotron sources are in the subsecond range. Beam sizes smaller than $100 \times 100 \mu m^2$ are available.

The measured quantity of the scattering experiment is the intensity as a function of the scattering angle $2\theta$. For good angular resolution the primary beam divergency needs to be small and the resolution of the detector must be high. A simple method to reduce the divergency of the primary beam is to use a collimator made of two pinholes separated by the distance $c$ (collimation length). Increasing $c$ will reduce the divergency but also the intensity. Focusing optics are often used to achieve higher intensity. The angular resolution determined by the spatial resolution of the detector ($\Delta x$) and the distance $l$ between the sample and the detector can be evaluated for small angles according to $\Delta(2\theta) = \arctan(x/l) - \arctan((x + \Delta r)/l) \approx \Delta r/l$ with $x$ being an arbitrary distance between the positions of the primary beam and the scattered beam in the detector plane. Increasing $l$ thus increases angular resolution and assuming a fixed size of the beam stop extends the angular range to smaller scattering angles but reduces the largest detectable scattering angle due to the finite detector size.

When a simple counter tube is used as detector it is necessary to scan the detector plane by moving the detector step by step in the plane. Because of the extended part of the simultaneously detected solid angle, reducing the measuring times significantly, it is more convenient to use one- or two-dimensional position sensitive detectors (1D/2D detectors), such as gas filled delay line detectors, image plate detectors or CCD cameras. Such detectors are available for both X-rays and neutrons. When using 1D detectors the information of the radial intensity distribution with respect to the primary beam is lost. Furthermore one has to pay attention to the fact that the total intensity scattered to angles $\theta = d(2\theta)/2$ is distributed over the solid angle $d\Omega = 2\pi \sin(2\theta)d(2\theta)$ which for small scattering angles increases approximately linearly with increasing $2\theta$. The detected intensities can be corrected when assuming a homogeneous intensity distribution over $d\Omega$ which is in general not valid for powder samples with any texture or other interparticle ordering. Such particle arrangements can be investigated using 2D detectors. Detector images of an X-ray and a neutron small angle scattering experiment, respectively, on a tripalmitin nanodispersion are displayed in Fig. 9. The inhomogeneous radial intensity distribution which is correlated to the anisotropic particle arrangement in these dispersions can be seen clearly. When measuring these samples with a 1D detector it depends on the particular sample orientation whether the superstructure is visible at all or to what extent. In any case without further information the detected intensities are hard to analyze quantitatively.

For laboratory use the very compact setup of a Kratky camera is often used for SANS experiments. This camera type

![Fig. 9. 2D-detector images of a SAXS (left side, recorded at ID02 beam line, ESRF, Grenoble) and a SANS (right side, recorded at D11 instrument, ILL, Grenoble) pattern of tripalmitin nanodispersions. The tripalmitin concentrations were 19% for the SAXS experiment and 15% for the SANS measurement. The dispersion medium for the SANS experiment was prepared using D_2O instead of H_2O. For both images the first interference peaks due to the particle stacking are marked by black arrows. The (001)-Bragg reflections are marked by white arrows. The strong radial anisotropy of all interference maxima indicates the formation of large liquid crystalline domains of particles oriented in parallel. In the X-ray image the broad shadow of the beam stop for the primary beam is visible. The rectangular beam stop is visible in the center of the neutron image.](image-url)
uses a horizontally oriented line shaped beam profile (of about 1 cm length) which is well collimated vertically but less well horizontally. Because of the camera geometry it is possible to record scattering curves down to $s \approx 0.01 \text{ nm}^{-1}$. The diffraction patterns are however 'smeared out' to small $Q$-values because of the poor horizontal collimation of the beam. This effect becomes more and more crucial for decreasing scattering angles. A 'desmearing' procedure has to be applied to get the true scattering function. These procedures are based on the assumption that the investigated sample is a perfect powder sample, which means that the investigated particles are small and randomly oriented over the whole sample in the beam. In Fig. 10 an uncorrected SAXS pattern of the hexagonal liquid crystalline phase of the non-ionic surfactant dobanol 40 in water is displayed. Instead of the three expected reflections for a hexagonal phase (ratios: $1 : \sqrt{3} : 2$), indicated by vertical lines, many partly overlapping features are observed. The reason for this artificial pattern is that the liquid crystalline phase exhibits a structure with large homogeneous domains. Some domains are oriented such that they fulfill Bragg’s law and because the domains are large their scattering intensity is high. The detected scattering angles of these domains equal the expected value or are lower if Bragg’s condition is fulfilled for a horizontally divergent beam. Thus each peak of the detected SALS pattern belongs to one or more special liquid crystalline domains of the stick shaped sample. When including a horizontal slit collimation option such that the primary beam is limited to 0.5 mm in width the expected pattern can be observed (dashed line in Fig. 10). Now there is of course nearly no smearing but some peak intensities have nearly vanished. The same effect has to be expected for concentrated LNP dispersions. Therefore the authors of this article were not able to detect e.g. stacked lamellae of triglyceride dispersions using a Kratky camera.

3. Differential scanning calorimetry (DSC)

Since DSC is a well-known technique in pharmaceutical research and development only a short overview will be given over the basic principles of DSC measurements and instrumentation. For detailed information, the reader is referred to the specialized literature, e.g., several comprehensive textbooks explaining the measurement principles and their applications [50,51]. Concerning particularly the pharmaceutical field, the book by Ford and Timmins gives an exhaustive overview over the relevant thermal analysis techniques and applications [52]. Moreover, there are several introductory articles covering the principles of the measurement technique and their applications in the pharmaceutical area, e.g., [53,54].

Structural alterations of materials are accompanied by heat exchanges, e.g., uptake of heat during melting or emission of heat during crystallization. DSC is designed to measure these heat exchanges during controlled temperature programs and allows to draw conclusions on the structural properties of a sample. It should be borne in mind, however, that, although
DSC is able to monitor and quantify even minute thermal events in the sample (depending on the sensitivity of the instrument, of course) and to identify the temperatures at which these events occur, it is a technique which does not directly reveal the cause of a thermal event. The exact nature of the thermal transitions has to be determined with complementary methods such as microscopic observations, thermogravimetry, X-ray diffraction or spectroscopic techniques to distinguish, for example, between melting, polymorphic transitions, loss of water from hydrates or decomposition of the substance. In common DSC investigations the respective sample is heated or cooled at a controlled rate and the heat flow into or out of the sample is monitored in a quantitative way. Although isothermal measurements are, in principle, also possible with conventional DSC equipment, they are not commonly performed, mainly since such measurements usually require a very high sensitivity of the instrument and thus specialized equipment (microcalorimetry).

There are two principal types of instruments used for DSC investigations, heat flux DSC and power compensation DSC (Fig. 11). Both contain two identical sample holders which are heated (or cooled), usually in a linear way. One sample holder contains a crucible filled with sample, the other a reference crucible. Under ideally symmetric conditions (perfectly symmetric instrumental setup and identical properties of sample and reference) the temperature in both crucibles increases at the same rate during a heating program and there is no temperature difference between sample and reference as long as no structural change occurs in the sample. When the sample undergoes a structural change, as for example a melting transition, it takes up energy, i.e. heat, from the system. As during the course of the transition all energy provided by heating is used for the transition, the sample temperature does not increase any longer. This leads to a temperature difference between the sample and the reference crucible which continues to follow the instrumental heating program. In heat flux DSC, this temperature difference is measured and converted into a heat flow signal through a calibration procedure. In power compensation DSC, where the two sample holders (microfurnaces) are supplied with individual heaters, the detection of any temperature difference between the furnaces leads to an immediate adjustment of the heating power in such a way that the temperature is kept (almost) identical in both furnaces. The additional (or reduced) heating power is directly correlated with the heat flow into or out of the sample. Adequate calibration is, however, required also for this measurement technique. After the calibration procedure, there are, in principle, no significant differences in the results obtained from the two different measurement techniques in routine experiments (although the experimental results may depend significantly on the sensitivity and resolution of the respective instrument). By convention, endothermic events are displayed as upwards signals in power compensation DSC and as downwards signals in heat flux DSC.

The signal obtained in DSC measurements is the heat flow as a function of time or temperature. For first-order transitions, like melting, crystallization or polymorphic transitions, signals of peak-like shape are observed (Fig. 12). Integration of the signals provides the heat exchanged during the transition. Glass transitions (which are, however, less important with respect to the characterization of LNP dispersions) are characterized by a step-like change of the baseline position. As an additional component, DSC curves usually show a deviation of the baseline from the zero position which, in addition to certain instrumental parameters, reflects the differences in heat capacity between the sample and the reference, which is usually an empty crucible. While this deviation is relatively small when the sample consists of small amounts of organic material (e.g., bulk fats) it may be quite large when aqueous dispersions are measured due to the high sample mass required and the large heat capacity of water (Fig. 12). In most cases, this deviation is irrelevant for the measurements as it can simply be subtracted.

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Fig. 12. Comparison of the DSC melting curves of trimyristin as raw material and as colloidal dispersion (10% trimyristin, 2.4% soybean lecithin, 0.6% sodium glycocholate in an aqueous phase containing 2.25% glycerol and 0.01% thiomersal).

Fig. 13. DSC heating curves of pure indium showing the influence of heating rate on the peak maximum temperature ($T_{\text{max}}$). The influence of the heating rate on the extrapolated peak onset temperature ($T_{\text{onset}}$) is much smaller. All curves were recorded with the same temperature calibration.

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4 Temperature modulated DSC, where the linear heating or cooling rate is superimposed with a sinusoidal or other type of temperature modulation, has not yet been used for the study of LNP dispersions and will thus not be covered here.
In specific applications, it may, however, be useful to balance the heat capacities between sample and reference by filling the reference crucible with an adequate amount of inert material. Some instruments, in particular those for high sensitivity investigations, even require this kind of heat capacity balancing.

For pure substances, the temperature of a first-order transition can be derived in a straightforward way as the extrapolated onset temperature of the respective thermal event of the DSC curve (Fig. 13). The extrapolated onset temperature does only little depend on the experimental conditions and influences can be corrected for by calibration. In contrast, other characteristic points of the transition, in particular the peak maximum temperature, highly depend on experimental parameters: Due to the thermal lag of the instrument, the use of higher heating rates will shift the maximum of the signal to higher temperatures (Fig. 13). For the same reason, the peak maximum temperature also tends to shift slightly to higher temperature with increasing sample mass. As outlined in more detail below (cf. Section 4.3), the determination of representative melting temperatures may be much more problematic for dispersed materials, in particular when the particle sizes are in the nanometer range. For such samples, the shape of the resulting transition frequently deviates significantly from that of a “neat” signal of a pure bulk substance, often making the determination of a meaningful onset temperature impossible. The shapes of signals that correspond to the crystallization of the dispersed particles upon cooling are usually less complex and an extrapolated onset temperature can, in most cases, be derived without problems. For exothermic transitions the onset temperature is a much more realistic value than the peak maximum temperature since the sample temperature during the transition is not very well controlled due to the evolution of heat as a result of crystallization [55,56].

With modern DSC equipment the thermal behavior of samples can be monitored between subambient temperatures (depending on the cooling device) and several hundred degrees centigrade. Nitrogen or helium is used as purge gases to avoid reactions with the atmosphere. For measurements in the lipid nanoparticle field, the temperature range between about −60 °C (for freeze-drying applications) and ∼350 °C (highest melting points of organic substances) is the most interesting, with particular regard to the temperature range of liquid water. Typical scan rates are between 1 and 10 °C/min although, in particular, lower rates may be used for specific applications or in specialized equipment. While low scan rates are preferable in terms of peak resolution and investigation of the sample close to equilibrium, high scan rates increase the sensitivity of the measurement as they lead to the exchange of heat within a comparatively short time period. It should be borne in mind that the scan rate may also influence the course of temperature-related processes within the sample. For example, high scan rates can suppress gradual transformation of metastable triglyceride modifications upon heating (cf. Section 4.4) and will lead to the detection of lower crystallization temperatures upon cooling than the use of slow cooling rates.

In conventional DSC instruments calibration of the temperature scale and the determined heat of transition is carried out on a regular basis usually using reference substances of high purity [50]. The most common calibration substance is indium (m.p. 156.6 °C) which is suitable for temperature as well as for heat (peak area) calibration. Other metallic substances like tin (m.p. 231.9 °C), lead (m.p. 327.5 °C) or zinc (419.5 °C) can be used for the generation of additional calibration points. Unfortunately, the comparatively high melting point of most metals is out of the temperature calibration range of highest interest for the investigation of aqueous lipid dispersions. As the only relevant exception is gallium (m.p. 29.8 °C) different organic materials or water (m.p. 0.0 °C) may be included in the calibration procedure to cover the whole temperature range relevant for the study of lipid nanoparticles. For accurate measurements, calibration should be performed at the same scan rate as employed for the measurements (unless a significant influence of the scan rate on the results obtained is ruled out by comparative investigations at different scan rates).

4. Applications of DSC and X-ray diffraction for the characterization of LNP dispersions

4.1. Some considerations on sample preparation

As outlined in more detail below, LNPs often have very peculiar properties which are due to their dispersed state and the small particle size. In order to retain these properties during investigation care has to be taken not to induce alterations by sample preparation procedures. If at all possible the dispersions should thus be investigated in their native state. Modern sensitive DSC instruments are capable of detecting the crystalline lipid phase transitions at the typical concentrations in LNP dispersions of about 1–10% or even below. Sensitivity may become an issue e.g. in the investigation of minor compounds of the particle composition or for phase transformations which involve small amounts of energy (for example, liquid crystalline phase transitions). Moreover, investigations of highly diluted samples can be problematic in particular also for X-ray investigations. If some kind of sample modification is inevitable prior to investigation, e.g., in the case that very diluted dispersions have to be concentrated to overcome problems with instrument sensitivity, the particle properties must not be changed by the sample preparation procedure, especially in terms of physical state and particle size. Against this background, drying of samples which is sometimes performed for analytical purposes is particularly critical. Drying may lead to pronounced alterations of sample properties as result of temperature effects (particularly upon freeze-drying) but also simply due to the loss of the dispersion medium. The effects may, for instance, concern transition temperatures, melting enthalpy and polymorphism as, e.g. reported in [48,57–59] and illustrated in Fig. 14. In cases where drying is inevitable the potential influences of the sample preparation procedure on the results obtained need to be carefully considered upon data interpretation.

4.2. Observation of general thermal phenomena in LNP dispersions

Probably the most obvious, easiest and most frequently used application of DSC in the study of lipid nanoparticle dispersions is
the investigation of the physical state of the dispersed particles via the detection of characteristic melting or transformation endotherms upon heating (or the absence of these endotherms, respectively). Moreover, the thermal behavior of the nanoparticles upon cooling is also conveniently studied with this method and the results may provide valuable information for the layout of potentially required thermal treatment after processing steps involving heat treatment [43,46,47,60]. The necessity to pay special attention to the physical state of LNPs arises from the fact that the properties of the lipid matrix materials may change significantly when they are brought into the colloidally dispersed state. For example, even when crystalline lipids are used for the preparation of LNP dispersions, the lipid particles are not necessarily present in the solid state after processing, in particular when the preparation process is carried out in the heat such as during melt homogenization [46,47,58,60–64]. This is due to pronounced supercooling in the dispersed state, a general phenomenon not only observed in lipids [61,65]. Considerable supercooling of the melt prior to crystallization is already often observed in bulk lipids, such as in triglycerides or cholesterol esters [47,64]. In nanodispersed lipids, due to the absence of particulate impurities (which promote crystallization of the bulk material) in the vast majority of the droplets crystallization is usually more retarded and occurs at lower temperatures than in the bulk lipids [9,47,60,61,64,65]. For instance, colloidal dispersions of saturated monoacid triglycerides may crystallize at temperatures about 20 °C lower than the bulk material leading to a difference between the melting maximum and the onset of crystallization of up to almost ~50 °C [47,49,60,66]. In other types of lipid materials, for example complex glyceride mixtures or paraffins, the supercooling effect is, however, less pronounced [67–69]. The confirmation of the desired physical state of the matrix lipid is thus of crucial importance for the development of nanoparticles based on solid lipids. In most cases, the desired state of the particles will be solid or at least partially solid (in some oil-containing solid LNPs). For special applications, however, also the state of a supercooled liquid melt may be aimed at, for example to achieve particular carrier properties [61] or to increase the bioavailability of highly lipophilic drugs [70]. Recently, also the formulation of nanoparticles based on supercooled smectic liquid crystals consisting of cholesterol esters has been proposed.

Fig. 14. Effect of air drying on the thermal behavior of triglyceride dispersions. Left: Trilaurin dispersion (10% trilaurin, 6% tyloxapol in an aqueous phase containing 2.25% glycerol and 0.01% thiomersal) prepared by melt homogenization. The original sample is an emulsion of supercooled liquid droplets which do not lead to a thermal event upon heating. After air drying an equivalent amount of dispersion at room temperature the resulting thin film displays a pronounced melting event. Right: Tripalmitin dispersion (10% tripalmitin, 10% tyloxapol in an aqueous phase containing 2.25% glycerol and 0.01% thiomersal; scan rate 2.5 °C/min). The original dispersion displays a structured melting event due to its small particle size (PCS z-average 60 nm, polydispersity index 0.20). The structuring is completely lost after air drying the sample.

Fig. 15. Small and wide angle X-ray diffraction patterns of cholesteryl myristate in bulk and in colloidal dispersion (5% cholesteryl myristate stabilized with 5% polyvinyl alcohol). Bulk: crystalline powder (a) at 20 °C and smectic mesophase (b) at 60 °C. Dispersions stored at 4 °C (c) and at 23 °C (d) and measured at 20 °C. The graphs of the bulk material and the dispersions are not on the same (linear) intensity scale. The smectic phase is characterized by a pronounced small angle reflection and the absence of wide angle reflections whereas the crystalline material displays reflections both in the small and in the wide angle range (from [64] with permission).
as a new type of colloidal lipid carriers [64]. All these dispersions are characterized by the presence (in the case of crystalline or liquid crystalline particles) or absence (supercooled melts) of characteristic thermal transitions in the DSC heating curve allowing to draw conclusions on their physical state. These conclusions may be confirmed by additional X-ray investigations which are particularly helpful in the case of liquid crystalline nanoparticles (Fig. 15) the thermal transitions of which can be very small. Simultaneously, information on the position of the melting and crystallization transitions can be collected. Such information is often required for a rational layout of the preparation process, in particular, to determine adequate cooling conditions to crystallize the particles from their melted state (or, on the other hand, to find out lower critical temperatures for the development of stable supercooled systems).

4.3. The effect of the small particle size on the thermal behavior

When comparing DSC thermograms of bulk lipids and corresponding solid LNP dispersions differences in shape and position of the signals are usually observed: The endotherm of the nanoparticles is broadened and shifted to lower temperatures (Fig. 12). In addition to possible effects of the adsorbed emulsifier molecules, an effect of the particle size on the melting temperature is to be expected for all nanodispersed materials according to the Gibbs-Thomson equation [71]:

\[
\ln \frac{T}{T_0} = -\frac{2\gamma_{sl} V_S}{r\Delta H_{fus}}
\]  

(11)

\(T\) Melting temperature of a particle with radius \(r\)

\(T_0\) Melting temperature of the bulk material at the same external pressure

\(\gamma_{sl}\) Interfacial tension at the solid–liquid interface

\(V_S\) Specific volume of the solid

\(\Delta H_{fus}\) Specific heat of fusion

This effect is reflected in a decrease in melting temperature for a particle of given size compared to the bulk material and becomes particularly pronounced in the lower nanometer size range. Because LNP dispersions are usually more or less polydisperse the melting transition is not only shifted to lower temperatures but is also broadened since the fractions of different particle sizes melt at different temperatures. For dispersions containing particles with fractions of specific, but distinctly different dimensions even the occurrence of a series of sharply separated melting events can be observed. Such behavior has been described for small size dispersions of saturated monoaic triglycerides in the stable \(\beta\)-modification [29,30,33,49] (Fig. 16, cf. Section 2.3.1). These particles represent thin platelets which can only exist in thicknesses corresponding to the multiples of a single molecular triglyceride layer (Fig. 17). As the thickness of a triglyceride monolayer (e.g., 4.06 nm for the \(\beta\)-form of tripalmitin) is relatively large...
compared to the overall thickness of the triglyceride nanoplatelets (which may consist of only several molecular layers) the steps in particle thickness are quite pronounced. The thickness distribution of the particles then leads to the occurrence of a structured melting event in which each peak represents the melting of a fraction with specific particle thickness [29]. The phenomenon becomes less pronounced with increasing complexity of the dispersed material, probably since complex lipid mixtures are unable to form particles with defined gradations in thickness thus producing only a diffuse, broad melting event with comparatively low transition temperature [30] (Fig. 16).

Other effects observed with decreasing particle size in colloidal solid triglyceride dispersions were a decrease in melting enthalpy (as also observed by [66]), an increase in the rate of polymorphic transitions as well as the occurrence of unusual reflections in small and wide angle X-ray diffractograms. Although a small decrease of the crystallization temperature with decreasing particle size has also been reported for colloidal triglyceride nanoparticles this effect was much less pronounced than the influence on the melting temperature (smaller than 2 °C for nanoparticles with PCS mean particle sizes between about 65 nm and 500 nm) [30].

The particle size related broadening and structuring of the melting transition in LNP dispersions significantly complicates the interpretation of the DSC curves. As found in investigations on the behavior of triglyceride nanoparticles, in certain cases the depression of the melting temperature due to the particle size effect may be so large, that the melting transition could be confused with that of a lower melting polymorphic form [30,60].

The interpretation of melting curves of lipid materials with more complex composition (which can already be very demanding for the bulk state) will be particularly complicated if pronounced influences of the particle size occur. In addition, the broadening of the DSC melting endotherms in nanoparticle dispersions often impairs the correct integration of the signals (as does their generally small size and sometimes also wide melting range due to other effects like complex composition and/or polymorphic behavior of the matrix materials). Larger errors compared to investigations on bulk materials have, therefore, to be taken into consideration upon quantitative evaluation.

As a result of the particle size effect, it is often difficult to define a characteristic melting point of the nanodisperse materials. The melting endotherm reflects melting of a huge array of particles each of which behaves individually (e.g., a 10 mg DSC sample of a 10% glyceride dispersion with a particle size of 150 nm contains in the order of 10^{11} nanoparticles). In principle, one would need to define the melting point of each single particle which is, of course, impossible in practice. An extrapolated onset value can often not be determined from the melting curves in a useful way since the shape of the curves deviates quite significantly from that of a ‘neat’ signal of a pure bulk substance unless the particles are rather coarse. As an alternative, the peak maximum temperature is often used. This parameter has the advantage of being also accessible for complex melting events with shoulders or side maxima, resulting, e.g., from polymorphic transitions or melting in fractions. A potential influence of the experimental conditions on the peak maximum temperature needs, however, to be taken into consideration (cf. Section 3). For very complex curves, e.g. for those with several maxima, the assignment of a single characteristic temperature does not appear to be useful and other parameters (positions of different peaks, span of the transition or display of the whole curve) may be more appropriate.

4.4. Polymorphism and aging phenomena

An important point to be considered in the characterization of LNP dispersions is the potential occurrence of different polymorphic forms. Polymorphism is commonly found in lipid materials and thus also in the components used in lipid nanoparticle dispersions, for instance, glycerides or fatty acids. The different molecular packing in the polymorphic modifications is reflected in differences of physical properties like the melting points and enthalpies. For simple lipids, like the saturated monoacid triglycerides or fatty acids, the properties of the different forms in the bulk are well-known [73–79] and can also be found in the nanodispersed materials. For other lipid materials like, e.g., complex glyceride mixtures (e.g., Witepsol, Softisan, Compritol) or waxes (e.g., cetylaluminate), which are also frequently employed as matrix materials in LNP dispersions,
published specific reference data is rare or even unavailable. For such materials (but sometimes also for simple lipids due to the particle size related distortion of the melting transition) the correct assignment of the polymorphic forms in DSC curves can be a major challenge. A sound interpretation of more complex melting curves thus requires information from complementary investigations, in particular by X-ray diffraction which allows more unambiguous assignment of the polymorphic forms and may also help to detect processes such as phase separations.

Glycerides occur in three major polymorphic forms, α, β′ and β (in order of increasing thermodynamic stability) which are characterized by different subcell packings of the lipid chains, different angles of tilt of the lipid chains with respect to the molecular glyceride layers and different densities. The different subcell packings lead to characteristic wide angle X-ray reflections enabling their identification (Fig. 18). For saturated monoacid triglycerides the α- and β-forms are frequently observed in the bulk state as well as in colloidal dispersions [31,46,81,82] whereas the β′-form usually occurs only under special conditions [84]. The β′-modification is, however, frequently observed in more complex triglycerides and triglyceride mixtures and in compositions containing larger fractions of partial glycerides (e.g., in many hard fats or glyceryl behenate) where it often represents the storage stable form [46,48,85]. The presence of the β′-form also in colloidal particles has been confirmed by X-ray diffraction on glyceryl behenate nanoparticles [44,48,68]. Similar wide angle diffraction patterns were also found for nanoparticles containing solid paraffin and cetyl palmitate [45]. Moreover, for complex triglyceride mixtures like hard fats an intermediate β-form (βi) has been described [86,87] which has also been observed in corresponding LNP dispersions [82,46].

The polymorphism of the major glyceride modifications is of the monotropic type, i.e. the form with the highest melting point is thermodynamically stable over the whole temperature range. Metastable polymorphs may thus transform into a more stable form already below their respective melting point, e.g., simply on storage. For instance, in saturated monoacid triglycerides the metastable α-modification is usually formed upon crystallization from the melt. Transformation into the stable β-form cannot only be induced by heating the system to the melting point of the α-modification but also occurs below this temperature upon aging of the sample. The kinetics of polymorphic transition from the α- into the β-form below the α-melting temperature depend on the type of triglyceride (shorter-chain triglycerides undergo the transition more easily) and the storage temperature (storage temperatures more close to the α-melting point will lead to more rapid transitions). These principles, which were originally established for bulk triglycerides [88] have also been observed in triglyceride nanoparticles [47,83].

LNPs prepared on the basis of fatty acids have less intensively been studied with regard to their solid state structure than glyceride based nanoparticles. Even saturated fatty acids like stearic and palmitic acid which are usually employed for the preparation of the corresponding systems can exist in four different polymorphic forms termed A, B, C and E [78,79,89]. All forms can be obtained upon crystallization from solution but only the C form grows upon crystallization from the melt. This form has also been found with DSC and X-ray diffraction in (dried) stearic acid nanoparticle preparations [90–93]. Although the B form is the stable modification of stearic acid below 32 °C [78,79] this form has only rarely been found in nanoparticulate material based on this substance, for example as a result of interaction with alcohol cosurfactants or drug incorporation [90–93]. The structure of the B and the C form is quite similar. Both forms have an orthorhombic subcell (similar to the β′-form of glycerides) and mainly differ in their angle of tilt (Fig. 19). They can be distinguished by their different melting points (with that of the C form being higher). In X-ray diffraction, the two forms can be identified by differences in the long spacings (corresponding to the distances between the single molecular layers) ([90,91,94]) whereas the wide angle reflection patterns for both forms are very similar due to the same type of subcell packing.

With DSC, the polymorphic status of a sample can be checked and the influence of different parameters such as storage time and temperature or interactions between the components of the dispersion can be studied this way. When investigating the presence of metastable polymorphs it needs to be taken into consideration that the sample properties may be

![Fig. 19. Schematic representation of the B and C form of fatty acids (modified after [76]).](image)

![Fig. 20. Influence of heating rate on the shape of the melting curve of a freshly crystallized tristearin dispersion (10% tristearin, 2.4% DPPC, 0.6% sodium glycocholate) and the ratio of the polymorphic forms detected. The metastable form originally present in the sample can only be reliably detected at high heating rate. At low heating rates, transformation into the stable β-form occurs during the heating run. The curves have been normalized with respect to the heating rate; the structuring of the β-transition is due to particle size effects.](image)
altered by the heating program of the thermal analyzer in the case of monotropic phase behavior of the matrix lipids. Metastable polymorphs may thus transform into more stable ones during analysis. Such transformations are sometimes reflected by distinct recrystallization events in the DSC curve but can also proceed very gradually and may then escape observation (Fig. 20). Obviously this would lead to an underestimation of the presence of metastable form. As gradual transformations are particularly observed at slow heating rates, high heating rates should be used to limit this problem in the analysis of polymorphic samples. Unfortunately, using higher heating rates will decrease resolution of multiple signals so that a compromise may be necessary. Methods operating at constant temperature like X-ray diffraction are advantageous in this regard. Although in principle well suited for this purpose, X-ray diffraction has so far not evolved into a routine method to study gradual polymorphic alterations of lipid nanoparticles in a quantitative way, probably because quantitative data is more difficult to obtain by this method, particularly for aqueous dispersions.

The correct, quantitative determination of the ratios of the different polymorphic forms by DSC is often difficult in practice due, for instance, to the superimposition of thermal events, particularly for complex matrix compositions. In such cases, following simply the evolution of the overall transition enthalpy in dependence on the parameters of interest, e.g., in dependence on storage time, may be a pragmatic solution [83, 95]. Often, this enthalpy is normalized to the melting enthalpy of this recrystallized fraction not to that of the bulk material the exact concentration of matrix lipid in the processed dispersions may deviate from that of the primary preparation of melt-homogenized samples or during storage of dispersions of supercooled droplets under inadequate conditions) should lead to an increase in ‘crystallinity’. In contrast, an increase in melting enthalpy due to polymorphic transitions should not be considered as reflecting an increase in crystallinity as the less stable polymorphic forms are also crystalline even though their melting enthalpy is lower. Even if the fully crystallized raw material in the stable polymorphic form is regarded as the reference for normalization, relevant reference values are sometimes difficult to obtain. Polymorphic transitions generally occur more rapidly in lipid nanoparticles than in the bulk state [49, 66, 82, 95] as illustrated in Fig. 21. Some materials (like certain hard fats) even transform into a more stable polymorphic form than is observed in the bulk material when they are dispersed into colloidal particles as confirmed by X-ray diffraction [46, 66]. Also glyceryl behenate nanoparticles have been observed in a more stable form than their bulk material under certain conditions [48]. In such cases, using the melting enthalpy of the raw material as reference value may obviously lead to a ‘degree of crystallinity’ higher than 100% in the nanoparticles. In some cases, the crystallization index does not refer solely to the crystallizing lipid but to the whole matrix lipid used for particle preparation, even if it contains lipid material that is not expected to crystallize in the particles, e.g., lecithin [66, 85]. This procedure leads to maximal ‘recrystallization indices’ below 100%. Low values may also result from the fact that the melting enthalpy in small LNP's is lower than that of the bulk material. This has been attributed to the contribution of interfacial effects [30]; a potential influence of the emulsifier layer on the enthalpy values has not yet been investigated. Moreover, for an accurate determination of the recrystallization index with reference to the heat of transition of the bulk material the exact concentration of matrix lipid in the dispersion has to be known in order to provide a correct basis for normalization. Although the concentration of matrix lipid in the processed dispersions may deviate from that of the primary composition used for preparation due to, e.g., evaporation of water during processing in the heat or dilution with process water in some homogenizers, determination of the lipid content in the final formulations is usually not performed. In some special cases, however, a reasonable estimate of the fraction of crystalline material may be possible even without knowledge of the sample concentration. For example, an approximation of the fraction of recrystallized matrix material in dispersions of supercooled melts or liquid crystals can be obtained by relating the melting enthalpy of this recrystallized fraction not to that of a bulk sample but to that of the same dispersion after enforced recrystallization of the dispersed materials, for example by cooling to very low temperatures [64, 100]. This procedure requires that enforced crystallization leads to the direct formation of nanoparticles in the fully crystallized state without formation of metastable polymorphs. Even when the material crystallizes into stable polymorphic forms under these conditions, freshly crystallized materials often have a lower melting enthalpy than after a certain time of storage. In these cases, use of this procedure leads to a slight overestimation of the recrystallized fraction [64]. One possibility to overcome this

![Fig. 21. Wide angle X-ray diffractograms of tristearin systems. Top: Thermally unstressed raw material; middle: raw material recrystallized from the melt and stored for 6 days at room temperature; bottom: dispersion (10% tristearin stabilized with 2.4% soybean phospholipid/0.6% sodium glycocholate) stored for 6 days at room temperature. The curves of the bulk materials and the dispersion are not on the same intensity scale ($s = 1/d = \sin \theta / \lambda$; $\theta$: scattering angle; $\lambda$: wavelength (0.15 nm)) (from [95]).]
problem could be the use of an artificially crystallized reference sample brought into the final state by aging from a fraction of the dispersion of interest.

A gradual increase in melting enthalpy over, e.g., several days or weeks of storage is not uncommon in dispersions of solid lipid nanoparticles as a result of polymorphic transitions and the formation of a higher degree of crystalline order [60, 83, 95–97]. Such processes are of high practical relevance since there are indications that the drug incorporation capacity of glyceride nanoparticles decreases with increasing packing order of the matrix lipids [46, 48]. Moreover, alterations in solid state properties lead to changes also in other sample properties, e.g., the particle shape [31, 95] which, in turn, might alter the rheological properties [32, 101]. Also the occurrence of colloidal instabilities in dispersions of glycerol behenate nanoparticles could be related to an increase in melting enthalpy and altered shape of the melting curve [96]. Jenning et al. reported on polymorphic transitions in glyceryl behenate nanoparticles due to the presence of electrolytes and under the conditions of a Franz cell drug release experiment as observed by DSC and X-ray diffraction [48].

4.5. Influences of dispersion composition

4.5.1. Dependence of the particle properties on the type of emulsifier

Emulsifiers used for dispersion preparation can influence the properties of the dispersed matrix lipids with regard to their crystallization behavior in different ways as established by DSC and XRD studies. In a screening of triglyceride nanodispersions stabilized with a large number of different surfactants it was found that there is a critical lower crystallization temperature range for a given triglyceride material in which the nanoparticles in most of the investigated dispersions crystallized [102]. This result is in good agreement with the assumption that crystallization in the dispersions occurs by homogeneous nucleation. Stabilization with certain surfactants leads, however, to an increase in crystallization temperature by several degrees. A closer investigation of this phenomenon [102] reveals that the surfactants leading to increased crystallization temperatures contain comparatively long, saturated alkyl chains whereas surfactants leading to crystallization in the lower critical temperature range possess short, branched or unsaturated lipophilic residues. The same principles also apply to the use of phospholipids as components of stabilizer mixtures for triglyceride nanoparticles [83]. The influence of some surfactants on the crystallization temperature is attributed to the induction of heterogeneous nucleation due to interaction of the lipid melt with the surfactant layer. The effect may be due to a templating effect of the orientated surfactant chains or even to the formation of a crystallized surfactant monolayer on the surface of the lipid particles as indicated by a ‘precrystallization’ event prior to the main triglyceride crystallization transition detected by DSC and X-ray diffraction investigations in most of the corresponding dispersions [83, 102]. Influences of the type of surface active components on the crystallization temperatures have also been reported from investigations in food science [8, 103–106]. In addition to the influence of the emulsifier on the crystallization temperature effects on the kinetics of polymorphic transitions were reported in studies on triglyceride nanoparticles [31, 60, 83, 97, 102]. The effects are related to the type of hydrophilic headgroup [102] but also to the structure of the lipophilic chain with long, saturated acyl chains delaying the transformation into the stable polymorph in comparison with shorter or unsaturated chains [83]. Besides DSC, time resolved X-ray diffraction studies are very helpful to study such kinetic phenomena, in particular for rapidly transforming systems (Fig. 22). Stabilization of triglyceride nanoparticles solely with bile salts like sodium cholate or sodium glycocholate also delays the transition into the stable form significantly [31, 60, 97, 102]. Moreover, the sole stabilization with sodium glycocholate has been found to induce a pronounced dependence of the internal particle structure of the metastable α-form, in particular the formation of ordered molecular triglyceride layers, on the crystallization conditions as reflected in DSC and XRD results [31].

For cholesteryl myristate nanoparticles in the supercooled smectic state effects of the emulsifiers on the stability against recrystallization and on the course of the crystallization event upon cooling have been observed by DSC. Fatty acid chain

Fig. 22. Time dependent evolution of the wide angle X-ray diffraction patterns of trimyristin nanoparticles during crystallization from the melt upon cooling. The dispersions consisted of 10% trimyristin stabilized with 2.4% phospholipid (either soybean lecithin (S100) or dipalmitoylphosphatidylcholine (DPPC)) and 0.6% sodium glycocholate. The time points given in the figure refer to the first appearance of a small angle reflection which was considered to mark the onset of triglyceride crystallization. The small reflection in the diffractogram of the DPPC-containing dispersion at 19.5 °C is due to the solidified chains of the phospholipid (from [83] with permission).
containing surfactants (e.g. phospholipids, fatty acids, sucrose ester) usually lead to a multiple crystallization event and to dispersions less stable against recrystallization of the lipid matrix than typical polymeric surfactants (e.g. poloxamer, polyvinyl alcohol) [107]. The occurrence of different polymorphic forms could be ruled out as the cause of the complex crystallization event in phospholipid/sodium glycocholate stabilized dispersions. Instead, the effect seems to be related to the presence of differently shaped particles in these dispersions [100].

4.5.2. Use of lipid mixtures as matrix materials

Mainly mixtures of two specific components for lipid nanoparticle preparation will be considered at this point because the influence of single components present in the multicomponent mixtures often employed as prefabricated matrix material for LNPs (e.g. hard fats) has hitherto only scarcely been studied. The admixture of a second glyceride to a given matrix material has been observed to lead to alterations in crystallization and melting behavior as well as polymorphic transitions. The crystallization temperature of the nanoparticles can either be increased or decreased by the admixture of a second lipid as observed in DSC measurements. For example, the addition of tristearin (C18-chains) to trilaurin or trimyristin (C12- or C14-chains, respectively) decreases the supercooling tendency of the shorter-chain triglycerides since the long-chain triglyceride induces nucleation at higher temperature [47]. Decreased supercooling has also been observed in mixed-chain triglyceride hard fats compared to a monoacid triglyceride with similar average chain length [46]. On the other hand, the addition of liquid, medium chain triglycerides to glycerol behenate reduces the crystallization temperature of the nanoparticles [68,108]. A reduced crystallization tendency upon storage was also observed for hard fat nanoparticles after admixture of medium chain triglyceride oil [99]. The crystallization tendency of supercooled smectic cholesterol ester nanoparticles could be modified by admixture of a second ester with lower crystallization tendency [107].

For glyceride nanoparticles prepared from mixtures of solid and liquid lipids also a depression of the melting point of the solid lipid has been reported with increasing concentration of liquid lipid [44,68,108,109] which clearly indicates the association of the two lipids within the single nanoparticles also after crystallization. The kind of interaction of the two matrix components cannot, however, be straightforwardly derived from the DSC data for this type of mixture. A melting point depression can, in principle, be caused by the formation of a solid solution or by eutectic behavior between liquid and solid lipid. For the glyceryl behenate/medium chain triglyceride mixtures under investigation in the mentioned studies a slight solubility of the liquid component within the solid matrix may be expected. The melting point depression has, however, mainly to be attributed to eutectic behavior since the results of other methods point to intraparticulate phase separation of the liquid from the solid lipid already at quite low oil concentrations which leads to oil localization at the surface of the solid lipid [23,24,44]. The presence of enclosed liquid oil compartments within a matrix of solid lipid has often been proposed for nanoparticles consisting of solid/liquid lipid mixtures [68,108–111]. There are, however, so far no convincing experimental data supporting this concept. A melting point depression of the solid compound due to admixture of a liquid component has also been reported for freeze-dried mixed tripalmitin/medium chain triglyceride and stearic/oleic acid nanoparticles prepared in analogy to nanoparticles from solid/liquid glyceride mixtures [110,112]. For freeze-dried formulations conclusions on the state of the mixture within the original nanoparticles are, however, even more difficult to draw than for the dispersion since the structure of the mixture may change during the drying procedure.

Also for binary mixtures of saturated monoacid triglycerides in the stable β-form, eutectic behavior would be expected in most cases due to the limited inter solubility of triglycerides under these conditions [81,113,114]. The extraction of phase diagrams of binary mixtures from DSC data is, in principle, a standard procedure (not only for lipid materials) which should allow assignment of eutectic/monotectic behavior, compound formation or the formation of solid solutions [115–117]. In an investigation on the melting behavior of a series of mixed trilaurin/tristearin and trimyristin/tristearin nanoparticles the interpretation of the DSC heating curves was, however, difficult. Complications arose from the very broad melting events, often with the occurrence of several additional peaks [47]. Both phenomena can at least partially be attributed to the particle size effect. This effect is hard to distinguish from an extended melting range expected for the higher melting compound in the two phase region of the eutectic phase diagram and makes the correct assignment of the major thermal events (melting of eutectic mixture and liquidus point) difficult. Although some conclusions were possible on the melting behavior of these nanoparticles (particularly the confirmation that the single particles still contained the triglyceride mixture) the phase behavior could not unambiguously be clarified in detail even with the help of additional X-ray data. X-ray diffraction did, however, allow to clearly distinguish a trilaurin and a tristearin-rich component in the respective nanoparticles and indicated a higher solubility of trilaurin in tristearin than vice versa.

Concerning the influence on polymorphic transitions, an enhancement of the transition into a more stable form has been observed by DSC and X-ray diffraction for glyceryl behenate nanoparticles after addition of liquid medium chain triglyceride oil [68]. This is in agreement with investigations on bulk material where increased transition rates have been reported for glycerides after addition of liquid oils [118]. Effects on the time course of polymorphic transitions have also been observed in nanoparticulate mixtures of the two solid triglycerides tristearin and trilaurin [47].

Recently, the admixture of lecithin has been proposed to alter the matrix properties of solid hard fat nanoparticles [66,85,119]. The melting and crystallization properties of the resulting nanoparticles were, however, still mainly governed by the hard fat component [66,85]. A more detailed investigation of the ultrastructure of these dispersions, amongst others with the aid of SAXS, pointed to the formation of a triglyceride-rich nanoparticle core surrounded by a shell rich in phospholipids rather than to the presence of lecithin in the particle core [120].
4.5.3. Investigations on the effect of drug loading

DSC and XRD are often used to examine the effect of drug loading on LNPs and to evaluate the state of the drug (crystalline, amorphous, molecularly dispersed) in the dispersions. When applying these techniques for the investigation of the state of incorporated drugs it should be kept in mind, however, that neither method is designed to find minor amounts of foreign crystalline substances in a carrier matrix. In X-ray diffraction, the detection limit depends much on the relative intensity and position of the signals of the component of interest compared to that of the carrier matrix (as well as stabilizers and other dispersion components). Even the occurrence of comparatively large signals arising from crystalline drug will be difficult to detect in a straightforward way when they are superimposed by the signals of the carrier matrix. At low drug concentrations, the signals may be completely lost in the baseline noise. Peak fitting procedures, e.g. by Rietveld analysis [121], may help to identify different crystalline components even in case of superimposed reflections but have hitherto not been used in the field of LNP research. As a first approach to evaluate whether the presence of crystalline drug (or other components of the mixture) would be possible to detect in the respective dispersions mixtures of the components in the same quantitative composition as present in the dispersion may be measured for comparison [43,122]. This approach still does not consider the fact that the signals from colloidal materials are broader and thus more difficult to analyze than those of bulk substances. As a further complication, the signals from dispersed material are generally less intense than those from bulk substances due to the low concentration of the particles in the dispersion medium (Figs. 15 and 21). Consequently, the samples are often dried prior to analysis which may, however, produce artifacts and cause further complications, e.g., due to the presence of cryoprotectants [122].

In DSC, the situation is even more complicated since the physical properties of the sample change during the temperature program. Therefore, the positions of the signals cannot necessarily be expected at the same positions as those of the pure raw materials. This will only occur when there is no miscibility of the corresponding substances, neither in the solid nor in the melted state. Usually, however, drugs incorporated into LNPs are soluble in the lipid melt so that an influence on the transition temperatures has to be expected, e.g., due to eutectic behavior. Drugs which were incorporated in the matrix lipid via dissolution in the melted lipid and are still associated with the particles after preparation may thus not give rise to a signal at their bulk melting point since, at this temperature, they may have redissolved in the lipid melt (very high heating rates may suppress this process to some extent). If at all, the melting transition of the drug will then be detected at lower temperatures. The detection of very small amounts of crystalline material can be difficult due to low concentration of drug as well as large width and the superimposition of transitions. The investigation of mixtures of the raw materials in the same ratio as used in the dispersions (as reported in [43,91,122–124]) may help to check for corresponding effects. Although the absence of drug melting transitions as, e.g. observed in [43,123,124], may point to a preferably amorphous or molecularly dispersed state of the incorporated drug the absence of clear drug melting events in DSC curves cannot be taken as final proof of a non-crystalline state of the drug in the sample. While the detection of a corresponding endothermic transition in an aqueous dispersion would be an unambiguous indication for the presence of crystalline drug (to the knowledge of the authors this has not yet been reported), the situation is less clear for dried samples (in which drug crystals might be formed during drying). Anyway, the occurrence of such melting signals (which have only scarcely been reported [93]) is usually taken as representative also for the situation in dispersion. In many cases it will be impossible to investigate the native aqueous dispersions for the presence of crystalline drugs as many of the substances melt above 100 °C and these temperatures are difficult to achieve in DSC measurements of aqueous samples. At the moment, drying of the dispersions seems to be the only alternative but the effects of this type of sample preparation remain to be investigated before its applicability can finally be evaluated.

An important question after drug incorporation into LNP dispersions is whether the drugs incorporated are indeed associated with the nanoparticles. Such an association may be reflected rather indirectly by an influence on the phase transitions of the matrix lipid. Although often regarded as confirmation of drug incorporation, the depression of the melting point of the matrix lipid is not necessarily an indication of drug incorporation into the lipid matrix or even into the crystalline lattice. For example, the presence of ubidecarenone, which, at higher drug loads, forms a separate liquid phase of supercooled drug material within single nanoparticles, causes a concentration dependent melting point depression of the solid lipid in triglyceride nanoparticles due to eutectic behavior between the two substances [43]. The melting point depression of cetyl palmitate in nanoparticles loaded with a high fraction of the liquid active tocopheryl acetate [125] is probably also due to this phenomenon. Although a depression of the melting point of lipid nanoparticles at lower (model) drug loads has sometimes been reported [43,57,93,122,126] small effects are difficult to detect in the dispersions as they may be superimposed, e.g., by the particle size effect. Moreover, the drug load in LNPs is often much below 10% so that distinct effects on the melting point cannot be expected. In addition to their influence on the melting temperature high loads of liquid substances may also result in a decrease of the melting enthalpy due to dissolution of fractions of the solid lipid in the liquid phase [43]. Effects on the melting enthalpy have, however, also been reported for lower drug loads [57,91,93]. Moreover, the presence of liquid or non-crystallizing active substances like ubidecarenone or tocopheryl acetate has been found to decrease the crystallization temperature of glyceride nanoparticles in a similar way as the loading with oil [43,127]. For smeectic cholesteryl myristate nanoparticles a decrease in the crystallization temperature was observed by DSC after loading the dispersions with 10% (related to the matrix lipid) of ibuprofen, etomídate and micamoxole [107]. For this type of particles, also a depressing effect of the drugs on the liquid crystalline phase transitions upon heating was observed which further confirms the association of drug with the nanoparticles.
With regard to polymorphic transitions, XRD revealed an enhancing effect of ubidecarenone on the transition of tripalmitin nanoparticles into the stable β-modification. Some effects of the presence of other drugs on the polymorphic transitions on triglyceride nanoparticles [127] as well as of model substances on the polymorphic behavior of dispersed stearic acid [91,93] have also been reported.

### 4.6. Solid LNPs in semisolid formulations

Although most DSC and X-ray characterization work on LNPs has so far been done on the primary dispersion formulations (or their dried analogues) some investigations on further processed nanoparticles have been reported. These studies mainly concern solid LNPs in semisolid formulations. Using DSC, the preparation-dependent distribution of the matrix lipid of paraffin nanoparticles in the different phases of a cream formulation was investigated [67]. By the same method, the presence and solid state of cetyl palmitate nanoparticles in gel and cream formulations was confirmed [125,128]. Also by DSC, an increased crystallization tendency of hard fat nanoparticles after incorporation into hydrogel formulations compared to the native dispersions was reported [99]. Jenning et al. [48] used X-ray diffraction to confirm the presence of β′-form in glyceryl behenate nanoparticles after incorporation into an oil/water cream.

### 5. Conclusion

DSC and X-ray/neutron diffraction and scattering techniques are indispensable tools for LNP characterization and provide many possibilities to derive information on the properties of the dispersed particles. Although the use of these techniques is straightforward in some cases, other applications in the field of LNP research require profound knowledge on the physico-chemical properties of the dispersions as well as on the specificities of the respective instrumental technique. Detailed knowledge on the analytical procedures and their potential does, however, also allow to derive much more information on the sample properties than commonly obtained today. Although they do require a somewhat larger analytical effort particularly the scattering techniques which bear a large potential for the investigation of LNP dispersions that remains to be fully explored.

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### Reference


