3 Characterization of Solid Lipid Nano- and Microparticles

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CONTENTS

3.1 General Considerations 41
3.2 Particle Size 42
  3.2.1 Photon Correlation Spectroscopy 43
  3.2.2 Laser Light Scattering 44
  3.2.3 Characterization of Microparticulate Contaminations 46
3.3 Zeta Potential 46
3.4 Crystallinity and Polymorphism 47
  3.4.1 DSC 49
  3.4.2 X-Ray Diffraction 50
3.5 Particle Morphology and Ultrastructure of the Dispersions 53
  3.5.1 Transmission Electron Microscopy 53
  3.5.2 Scanning Electron Microscopy 57
  3.5.3 Optical Microscopy 57
  3.5.4 Atomic Force Microscopy 58
3.6 Interaction with Incorporated Drugs 58
3.7 Conclusion 60
References 61

3.1 GENERAL CONSIDERATIONS

To ensure the suitability of solid lipid nanoparticle dispersions for the intended type of application, and to enable a focused development of dispersions with specific properties, the characteristics of the dispersions have to be known in detail. A major point of interest is the question of sufficient colloidal stability of these thermodynamically labile systems. However, a detailed characterization of these systems is also very important when it is considered that the material properties of solid lipids, particularly the melting, crystallization, and polymorphic behavior of these lipids, may change dramatically when the substances are dispersed into particles in the lower nanometer size range. The behavior of lipid microparticles, which closely

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resembles that of the bulk material, is less peculiar in this respect. The characterization of lipid nanoparticle formulations is often not an easy task because of the small size and sometimes low concentration of the dispersed particles. Moreover, colloidal dispersions of solid lipids are extremely complex systems. Several types of particles often coexist in the dispersions, and the solid state of the particles generally allows more complicated processes (such as polymorphism and variations in particle shape) than, for example, the liquid state of emulsion droplets. Sophisticated experimental techniques are thus required to address the different questions concerning the structure and behavior of these systems. The aim of this chapter is to introduce the methods commonly applied for the characterization of lipid nanosuspensions and to give an impression of their capabilities as well as their problems. This chapter focuses on nanosized dispersions based on solid lipids, but it also includes some considerations about the corresponding microparticle field.

3.2 PARTICLE SIZE

Particle size is one of the most important characterization parameters for solid lipid nanoparticle dispersions, and parameters relating to particle size are consequently reported in all studies on these systems. Particle size determinations are predominantly performed to confirm that the desired colloidal size range has been obtained during preparation and that it is retained upon storage or further processing (e.g., during freeze drying or sterilization).

Moreover, particle size can significantly affect the material properties of the nanoparticles and is important for their interaction with the biological environment (e.g., as concerns their ability to pass fine capillaries or to leave the vascular compartment via fenestrations after intravenous administration). Particle sizing results are thus crucial parameters in the development and optimization of preparation processes as well as in the evaluation of dispersion stability. Particle sizing, however, has also been employed for other purposes: for example, to evaluate the size dependence of the nanoparticle matrix properties [1] or to obtain additional information on the particle shape [2,3].

Almost all particle size determinations of solid lipid nanoparticle dispersions are performed by light-scattering methods (some information on the particle size can also be obtained by nanoscale microscopic methods, which will be described in Section 3.5). In spite of the practical convenience with which values of particle size and size distributions can be obtained with commercially available equipment, the light-scattering methods used are not without problems. The particle size of solid lipid nanoparticles can be in a rather difficult range for particle size analysis with light-scattering methods — it is sometimes in the upper range of photon correlation spectroscopy and is usually at the lower limit of laser diffraction. Moreover, the conventional theories for deriving particle sizes from light-scattering data assume suspensions of particles that are all of the same type, homogeneous and spherical. Neither of these prerequisites may apply for dispersions of solid lipid nanoparticles, which often contain anisometric and sometimes even inhomogeneous particles. In many cases, the dispersions also contain other types of colloidal particles (such as micelles or vesicles) in addition to the solid lipid particles of interest. Moreover, the

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dispersions usually have to be diluted for particle sizing. Although this is usually not considered a practical problem, the redistribution of the components may alter the colloidal composition of the sample, and, in some cases, instabilities occurring on dilution can make a meaningful characterization of the particle size problematic. All these potential complications have to be taken into consideration when working with particle sizing methods. As a consequence, particle sizing results — or at least those obtained in routine analysis — should be regarded as approximations rather than as absolute values. Combining the information obtained from different particle sizing techniques (and sometimes also from visual inspection of the sample) can help the investigator to get a better impression of the "real" particle size range.

3.2.1 Photon Correlation Spectroscopy

The most widely used method to characterize the size of solid lipid nanoparticles is photon correlation spectroscopy (PCS). This method requires only very small amounts of sample and is rapid and easy to perform, and its range of operation (nominally between a few nanometers and a few micrometers) covers the relevant range for lipid nanoparticle suspensions. PCS analyzes the Brownian motion of the particles in the dispersion medium [4–7]. The randomly moving particles are irradiated with a laser beam, and the intensity of the light scattered from a small volume of the sample in a (usually) fixed or variable angle is recorded in dependence on time. The scattered light "flickers" as a result of the particle motion. Small particles lead to fast intensity fluctuations as a result of their high diffusion coefficient, whereas for larger particles, which move more slowly, the fluctuations are slower. The scattering intensity–time curve is analyzed via an autocorrelation function from which parameters relating to particle size and size distribution can be derived. The so-called *z*-average diameter (*z*-ave, sometimes also referred to as the effective diameter) and the polydispersity index (PI), as an indication of the width of the particle size distribution, can be derived directly from the autocorrelation function in a comparatively simple manner (method of cumulants) [8]. PCS particle sizes for solid lipid nanoparticles are usually reported using these parameters. The *z*-ave/PI values are quite robust and well suited to characterizing dispersions in a comparative way, provided that the PI is not too high, as this will preclude a meaningful interpretation of the results from the cumulant-based evaluation. Particle size results with fairly high (>0.3 to 0.4) PI values have occasionally been reported for solid lipid nanoparticle dispersions [9–14], but the corresponding parameter sets should rather be regarded as indications for "a very broad, heterogeneous dispersion" than as realistic parameters in an absolute way. The *z*-ave and PI are quite specific for this type of analysis and do not have much in common with the parameters normally used for the description of particle size distributions (such as volume or number diameter). The *z*-ave is intensity weighted and, as large particles scatter light much more strongly than smaller particles, gives a relatively large estimate of the particle size. Assuming, for example, a log normal particle size distribution, *z*-ave and PI can be "translated" into parameters characteristic of a monomodal intensity-, volume-, or number-weighted distribution [15]. In cases in which the PI is not extremely small, however, such a procedure may lead to highly artificial values because the

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real particle size distribution may not be monomodal at all. Moreover, a meaningful transformation into a number or volume distribution usually requires information on the optical properties of the particles (such as their refractive index) unless they are very small.

The extraction of more complex particle size distributions from PCS data (which is not part of the commonly performed particle size characterization of solid lipid nanoparticles) remains a challenging task, even though several corresponding mathematical models and software for commercial instruments are available. This type of analysis requires the user to have a high degree of experience and the data to have high statistical accuracy. In many cases, data obtained in routine measurements, as are often performed for particle size characterization, are not an adequate basis for a reliable particle size distribution analysis.

Because PCS relies on the determination of the particle diffusion coefficient, it is not a direct method for the determination of particle sizes. Information on the particle size can be obtained via the Stokes–Einstein equation

$$D = kT/3\pi\eta d \tag{3.1}$$

where D is the diffusion coefficient, k is Boltzmann's constant, T is the absolute temperature, η is the viscosity of the dispersing liquid, and d is the particle diameter. Determination of the particle size thus requires a well-defined and exactly known temperature during the measurement and requires information on the viscosity of the dispersion medium. Usually, a spherical particle shape is assumed for data evaluation. In contrast to the situation with latex or lipid emulsion particles, this assumption may not be justified for solid lipid nanoparticles, which frequently crystallize in a platelet-like shape [1,2,16–20]. Because the diffusion coefficient of anisometric particles is larger than that of a sphere of the same volume [21,22], a larger hydrodynamic diameter is observed in PCS for these anisometric particles compared with corresponding emulsion systems in spite of the volume contraction on crystallization (Table 3.1).

Although PCS is very reliable in giving characteristic particle size values for narrow, monomodal distributions of particles in the nanometer range, it is not an optimal method to provide detailed information on dispersions with broad or multimodal particle size distributions, particularly when these dispersions contain a considerable fraction of particles in the upper nano- or micrometer size range. The presence of large particles or aggregates may severely disturb the measurement. Removal of such particles, for example, by filtration or the use of inbuilt electronic "dust filters" is not recommended because it will produce misleading results unless the separated large-particle fraction is characterized separately. However, samples that appear problematic from visual inspection as a result of the presence of larger aggregates may sometimes give surprisingly "nice" results in PCS cumulant analysis.

3.2.2 Laser Light Scattering

For broader size distributions and dispersions that contain a considerable amount of particles in the upper nanometer and/or the micrometer range, laser light scattering

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TABLE 3.1
Examples of Differences in Photon Correlation Spectroscopy Particle Size as a Result of Different Particle Shapes in Lipid Nanosuspensions and Nanoemulsions

	Z-Average Diameter (Polydispersity Index)	
	Suspension (nm)	Emulsion (nm)
Trilaurin (Dynasan 112)	156 (0.16)	137 (0.15)
Trimyristin (Dynasan 114)	163 (0.13)	149 (0.13)
Tripalmitin (Dynasan 116)	165 (0.20)	129 (0.18)
Hard fat (Witepsol H42)	191 (0.12)	179 (0.13)
Hard fat (Witepsol W35)	145 (0.18)	129 (0.16)

Note: The samples (10% matrix lipid stabilized with 2.4% soybean phospholipid [Lipoid S100] and 0.6% sodium glycocholate) were obtained by high-pressure homogenization (Micron Lab 40, 5 cycles at 800 bars) above the melting temperature of the matrix lipid. The crystalline nanoparticles (obtained by cooling the particles to below 0˚C in the case of the trilaurin dispersion) were transformed into emulsion droplets by heating the dispersion above their melting temperature before measurement.

(LS; also referred to as laser diffraction) with an adequate instrument is probably a better choice than PCS for getting an impression of the particle size distribution (narrow, monomodal distributions can, of course, also be characterized by LS). A laser diffractometer determines the angular distribution of the light scattered from the dispersion on irradiation with laser light by an array of detectors [23]. The geometric distribution of the scattered light arising from a particle depends on its size: Large particles, compared to the wavelength of the laser light, scatter predominantly in a forward direction, whereas very small particles emit a more spherelike "cloud" of scattered light. Analysis of the angular intensity distribution of the scattered light thus gives information on the particle size. For the calculation of particle size distributions, iterative processes are usually applied to fit a model distribution to the experimental data. The instrument gives an estimation of the particle size distribution in addition to characteristic particle size values (e.g., mean, mode, median diameter, diameter at 90 or 99% of the distribution) in a comparatively short time. The technique is well suited, for example, to characterizing lipid microparticles [24–27].

Originally, LS instruments were constructed for the investigation of particles in the micrometer and millimeter size range only, using the Fraunhofer approximation (which is valid for particles that are very large compared with the wavelength of the laser light) for data evaluation. Modern instruments are also capable of using the Mie theory as a basis for data evaluation, and thus they are also theoretically prepared to evaluate the scattering pattern in the nanometer region. The scattering information provided by traditional experimental setups is, however, insufficient for an adequate evaluation of small particles in the nanometer range. Some modern LS instruments are, therefore, equipped with "submicron" features that aim at the investigation of

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nanometer-sized particle fractions in particular. Additional lamps with different wavelengths, wide-angle detectors, or special setups giving information on the interaction of the particles with polarized light provide complementary data on the nanoparticle fraction [28,29]. Such instruments have frequently been used for the characterization of lipid nanosuspensions [3,11,30–37]. As a drawback, applying this technique in the nanometer range requires knowledge of the optical parameters (the real and imaginary part of the refractive index at the corresponding wavelength) of the dispersed material. The calculated particle size distributions may depend highly on the optical model that is constructed on the basis of these parameters. Moreover, as in PCS, the models assume the particles to be spherical, which leads to further uncertainty in the results when nonspherical lipid particles are under investigation. For the characterization of dispersions that simultaneously contain particles in the nano- and micrometer size ranges, commercial instruments often apply data obtained from the application of different physical methods that require different theoretical models for evaluation. The experimentally and theoretically complex combination of these methods may cause additional uncertainties in the results.

3.2.3 Characterization of Microparticulate Contaminations

In particular, trace amounts of microparticles may be difficult to detect in solid lipid nanoparticle dispersions with light-scattering methods. The question of microparticulate contamination, which is of particular interest for dispersions developed with respect to parenteral administration, has hitherto been addressed only scarcely. Electrical zone sensing (the Coulter counter method) has been used to determine the absolute number of particles in the micrometer range for dispersions of lipid nanoparticles [11]. This method has also been applied for the particle size determination of solid lipid microparticles [38,39]. It is based on alterations of the electrical resistance of an aqueous salt solution within a pinhole when this pinhole is passed by a particle [40]. The technique was originally developed for counting cells but is also well accepted for the determination of particle size distributions in the micrometer range. In particular, for electrostatically stabilized colloidal particles, the need to be dispersed in a comparatively concentrated salt solution (e.g., 0.9% sodium chloride) is a major drawback, as the presence of ions may lead to the destabilization of the nanoparticles because of interference with the electric double layer. As an alternative, light-blockage or light-microscopic methods may be considered, though, except for a microscopic study in semisolid preparations, they hitherto have not been used for the characterization of suspensions of lipid nanoparticles with respect to microparticulate contaminations [35]. Although not suitable for the evaluation of particle size distributions of colloidal suspensions, light microscopy and optical imaging systems can be applied for particle size characterization of lipid microparticles [41,42].

3.3 ZETA POTENTIAL

Colloidal particles usually bear a surface charge as a result of the presence of ionized groups or of ion adsorption from the dispersion medium. These surface charges and the strength and extension of the electrical field around the particles play an important

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role in the mutual repulsion of nanoparticles and thus in their stability against aggregation. For colloidal drug carriers, the surface charge also has an impact on their *in vivo* behavior. Because the surface potential of the particles cannot be measured directly, the zeta potential ζ (electrical potential at the surface of hydrodynamic shear around the colloidal particles) is usually determined as a characteristic parameter for the nanoparticle charge [4,43]. For measurement, a dilute suspension of the nanoparticles is subjected to a weak electric field, and the mobility of the particles is commonly determined by laser Doppler anemometry. This technique is based on the evaluation of a frequency (Doppler) shift that is observed for the light scattered from the particles' motion in the electric field. As a result, the electrophoretic mobility μ (velocity of the particles/electric field strength) of the nanoparticles is obtained. For comparatively large particles in a weak electric field, the zeta potential can be derived from this value using the Helmholtz–Smoluchowski relation

$$\mu = \varepsilon\zeta/\eta \tag{3.2}$$

where μ is the electrophoretic mobility, ε is the permittivity, ζ is the zeta potential, and η is the viscosity of the dispersion medium.

In the study of solid lipid nanoparticles, zeta potential determinations have mainly been employed with respect to conclusions about the physical stability or instability, respectively, of the formulations during storage or on interaction with electrolytes or (simulated) biological fluids. Zeta potential measurements were, for example, used in the investigation of gel formation phenomena [16,31,33] and to assess different compositions with respect to electrolyte and pH stability [35,44]. When drawing conclusions about stability issues from zeta potential measurements, however, it has to be taken into account that surface charge may not be the only stabilizing mechanism, particularly when pegylated or macromolecular compounds are employed for stabilization, as they add a steric component. Investigation of the effect of different surface-active agents on the zeta potential can provide information on the interaction of the particles with surface-active agents [45–49]. The effect of variations in preparation procedure [50] as well as the potential influence of drug loading [11,49,51] or further processing, such as freeze drying or sterilization, on the zeta potential of solid lipid nanoparticles has also been studied [49,52]. In the development of particulate carriers with good adsorbing capacity for oppositely charged molecules, such as DNA or peptides, zeta potential investigations are a very important tool that allows optimization of the surface charge and investigation of the interaction of the particles with the molecules to be adsorbed [25,53,54]. In many cases, however, the zeta potential simply serves as a "standard" parameter to characterize the properties of the dispersions [14,55–61].

3.4 CRYSTALLINITY AND POLYMORPHISM

When preparing lipid nano- and microparticles from solid, crystalline raw materials, it is usually expected that the lipid matrix of the particles is or will become solid after the dispersion step. It has, however, turned out that some matrix materials do not crystallize easily in the colloidally dispersed state after processing in the heat

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as, for example, in melt-homogenization. Shorter chain triglycerides like tricaprin, trilaurin, or trimyristin are particularly problematic in this respect, but retarded crystallization has also been observed for dispersions of more complex glycerides [11,49,62–66]. Dispersions of such materials may require special thermal treatment after preparation to ensure the solid state of the particles. In addition to the lipid matrix composition, the solidification process can also be affected by the stabilizer composition or by the presence of a drug [67,68]. Because the expected advantages of solid lipid particles (e.g., modified release properties) essentially rely on the solid state of the particles, monitoring of the crystalline status is a crucial point in their characterization, particularly when novel compositions or preparation procedures are introduced.

Solidification of the particles may not be the final step in the formation process of solid lipid particles. Lipidic materials exhibit rich polymorphism [69,70], which may also occur in the dispersed state. In nanoparticles, the polymorphic behavior of the matrix lipids may, however, differ distinctly from that in the bulk material. Polymorphic transitions are usually accelerated in the nanoparticles compared with the bulk lipids [2,62]. In some cases, polymorphic forms not observable in the corresponding bulk materials were detected in lipid nanoparticles [1,65]. Because polymorphism can affect pharmaceutically relevant properties of the particles, such as the drug incorporation capacity [65], corresponding investigations should also be included in the characterization process. As long as polymorphic or other crystal-aging phenomena have not terminated, the particle matrix cannot be regarded as "static," and alterations of the particle properties may still occur.

Differential scanning calorimetry (DSC) and x-ray diffraction (XRD) are the techniques most widely used for the characterization of crystallinity and polymorphism of solid lipid particles. Although DSC is usually more sensitive in detecting crystalline material, XRD is much more reliable in determining the type of polymorph present in the dispersions because it provides structural data. In contrast, DSC can detect the type of polymorph only indirectly via the transition temperatures and enthalpies. Because these parameters may be different from those observed in the bulk material, particularly for small colloidal particles [1,62], assignment of polymorphic forms in DSC curves should be supported by x-ray data.

Modern DSC and XRD equipment is usually capable of analyzing lipid particles in their native, dispersed state, which is the preferred mode of investigation unless the final formulation is a dry product (e.g., in the case of microparticles or freeze-dried powders for injection). If problems with sensitivity arise, for example, when the samples are highly diluted — the samples may have to be concentrated before measurement. In this case, great care has to be taken not to change the properties of the particles during the sample preparation procedure (e.g., by causing an increase in particle size or by application of temperatures that may lead to phase transitions). Freeze drying or air drying of samples, for example, may lead to changes in transition temperatures, crystallinity, and polymorphism [11,71–73] and should, therefore, be avoided if possible. In some special cases, such procedures may be inevitable (e.g., drying samples to be checked for the presence of high-melting drug crystals by DSC [52,74]), but the potential alterations of the sample caused by the preparation technique have to be considered on data interpretation.

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3.4.1 DSC

In DSC the sample is subjected to a controlled temperature program, usually a temperature scan, and the heat flow to or from the sample is monitored in comparison to an inert reference [75,76]. The resulting curves — which show the phase transitions in the monitored temperature range, such as crystallization, melting, or polymorphic transitions — can be evaluated with regard to phase transition temperatures and transition enthalpy. DSC is thus a convenient method to confirm the presence of solid lipid particles via the detection of a melting transition. DSC recrystallization studies give indications of whether the dispersed material of interest is likely to pose recrystallization problems and what kind of thermal procedure may be used to ensure solidification [62–65,68,77].

DSC is also well suited to monitor physical changes: for example, those caused by polymorphism or an increase in crystallinity upon storage. In the case of polymorphic matrix lipids, the determination of an absolute value for the crystallinity requires the unambiguous assignment of the DSC transitions to the different crystal forms. Comparison with the thermal values described or observed for the bulk material may be inadequate for this purpose, particularly when very small particles are under investigation, as dispersing the matrix materials into the colloidal state may affect their melting behavior. A decreased melting temperature has frequently been observed, particularly in small-size lipid nanoparticles — in some cases to such an extent that confusion of the melting transition with that of a less stable polymorph could be possible [1,50,62]. Eutectic behavior with incorporated compounds such as oils or drugs may also lead to a shift in the peak positions [37,68]. Parallel XRD studies are very helpful in determining the type of polymorph present. If an absolute value for the crystallinity is not required (e.g., to simply monitor changes over storage), the value observed for the bulk material could be used as a reference even without knowledge of the crystalline form of the nanoparticles. In this case, "crystallinity" values above 100% have to be expected in cases in which the particles transform into a more stable polymorph than that present in the bulk material. The degree of crystallinity of solid lipid nanoparticles has been investigated with respect to its influence on a variety of application-related parameters such as gelation tendency, enzymatic degradation, or occlusive properties [32,78,79]. An increase in crystallinity during storage has been reported for particles prepared by melt homogenization, depending on composition [2,32,62]. The melting enthalpy of glyceride nanoparticles decreases with particle size [1]. When prepared from monoacid triglycerides, smaller nanoparticles (<150 to 200 nm) display a very peculiar size-dependent melting behavior with multiple transitions that are not caused by polymorphism, as confirmed by XRD (Figure 3.1). Such complications are not expected in lipid microparticles, which can be characterized with respect to polymorphism (depending on preparation procedure and composition) more easily with DSC by comparing the position and size of the transitions with those of the bulk material [39,80].

DSC has also been used to evaluate interactions with incorporated drugs. These interactions can be reflected in changes of the melting and recrystallization temperatures or in differences in melting enthalpy [52,68,81]. Direct evidence of the

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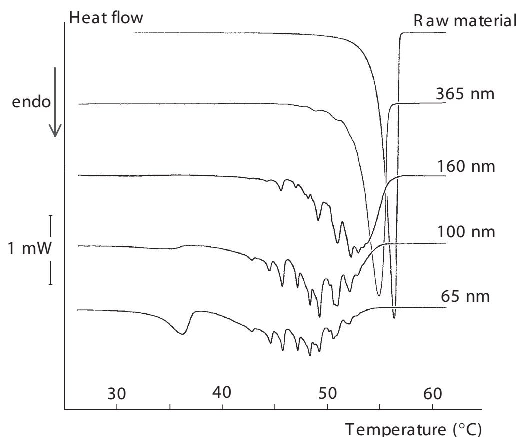

FIGURE 3.1 Differential scanning calorimetry melting curves (scan rate 0.04°C/min) of trimyristin dispersions (10% triglyceride stabilized with different concentrations of tyloxapol) with different mean photon correlation spectroscopy *z*-average diameters. The raw material was dispersed in an aqueous phase containing 1% tyloxapol. (Adapted from [1]. Copyright 2000, American Chemical Society. With permission.)

presence of crystalline drugs in the particles or dispersions as reflected by the occurrence of a melting transition has only scarcely been reported [26,82]. The absence of drug-melting transitions may point to a preferably amorphous or molecularly dispersed state of the incorporated drug [52,68,74], but the detection of very small amounts of crystalline material can be complicated by effects such as a large width and the superimposition of transitions.

The presence and state of solid lipid nanoparticles incorporated into semisolid formulations have also been investigated by DSC [77,83,84]. Using this method, de Vringer and de Ronde were able to draw conclusions on the preparation-dependent distribution of the matrix lipid of their particles in the different phases of a cream formulation [77].

3.4.2 X-Ray Diffraction

The interaction of a monochromatic x-ray beam with the crystal lattices in randomly oriented powder or suspension particles gives rise to a set of reflections that can be detected with a film, a moving counter tube, or position-sensitive detectors [85]. The resulting diffractogram, displaying the intensity of the reflections with respect to their angular position related to the incident x-ray beam, is specific for the crystal structure under investigation and can be used to identify substances or their different crystal forms. In the characterization of solid lipid nanoparticles, the major points of interest are usually the confirmation of the solid, crystalline state of the particles and the identification of the polymorphic form of the lipid matrix [10,37,65,68,73,81,86–88]. This can be done by comparison to literature data or to measurements of the corresponding bulk materials. The x-ray diffractograms of the

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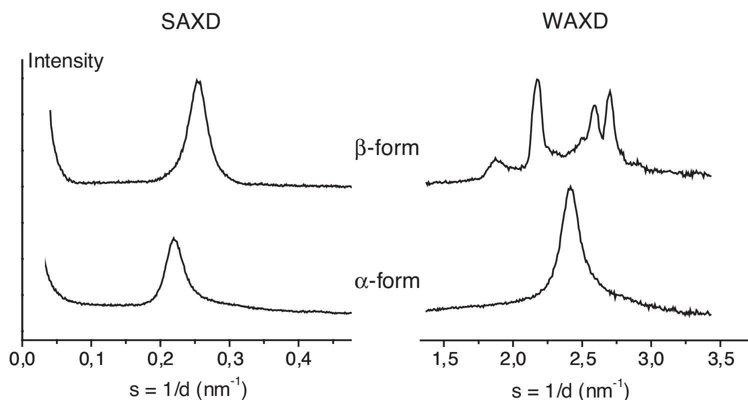

FIGURE 3.2 Small-angle (SAXD) and wide-angle (WAXD) x-ray diffractograms of tripalmitin nanoparticles in the α- and β-modification. s = scattering vector; d = d-value.

lipid matrix materials usually display only few major reflections characteristic of the packing of the alkyl chains. The lipids crystallize in lamellar organizations, with the alkyl chains packed side by side in different arrangements and oriented either perpendicular to or tilted toward the plane of the single layers. Typically, these arrangements display a strong small-angle reflection, indicating the repeating distance of the single layers. The packing of the alkyl chains within the layer is reflected in the occurrence of one or more reflections in the wide-angle region, where the reflections of crystalline drugs also are to be expected. As the wide-angle reflections for a specific polymorphic form are usually very similar within a given class of lipids, these reflections are particularly suitable for identifying the polymorphic form of the sample (Figure 3.2).

With the help of XRD, the differences in polymorphism between glyceride nanoparticles and the corresponding bulk material were clearly demonstrated [2,65,86]. Moreover, this method has been applied to evaluating the factors that affect the rate of polymorphic transitions in glyceride nanoparticles such as the type and chain length of the lipid, the presence of drug or liquid oil, the type of surfactant, or the particle size [1,64,65,67,68,89,90]. Microparticles were analyzed by x-ray diffraction to evaluate the influence of composition and preparation procedure on the resulting polymorphic form [39]. Temperature-dependent x-ray scans have proven to be very useful in addressing more complex questions such as the time course of polymorphic transitions during the crystallization process on cooling or the processes underlying complex DSC melting curves. For continuous scans, the use of synchrotron radiation as a source of intense x-radiation is usually necessary to minimize the exposure time for the single diffractograms and to provide the required time resolution (Figure 3.3).

The x-ray reflections of solid lipid nanoparticles are usually much broader than those of the bulk material as a result of the small particle size and, potentially, also of a decrease in crystalline order. Fine details may thus not be recognizable in the resulting, more diffuse diffractograms. Assignment of polymorphs may become increasingly difficult with the complexity of the material under investigation (e.g.,

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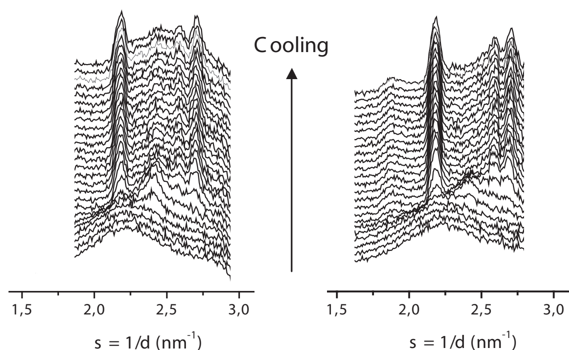

FIGURE 3.3 Evolution of the wide-angle x-ray diffraction patterns of differently stabilized tripalmitin dispersions (10% tripalmitin prepared with 3.2% soybean lecithin and 0.8% sodium laureate [left] or 2% soybean lecithin and 2% Tween 20 [right]) during the crystallization process. The samples were cooled with ~0.3°C/min, and the diffractograms represent the situation every other minute. The emulsion present at the beginning of the scan only displays diffuse scattering. The sample to the left retains a considerable fraction of α-modification beside the developing β-modification, whereas the scattering pattern of the dispersion to the right quickly transforms into that of the β-modification.

complex glyceride mixtures), particularly in cases in which there is also very limited knowledge about the structure and behavior of the bulk material.

On incorporation of ubidecarenone into tripalmitin nanoparticles, the presence of the drug was reflected indirectly in a change in the rate of polymorphic transitions and in differences in the width of the small-angle reflection, but not in the formation of signals caused by the crystalline drug, which was confirmed to be amorphous by complementary investigations [68]. In general, x-ray signals caused by the presence of incorporated drugs have usually not been reported for solid lipid nanoparticles [10,65,73]. This may point to the presence of molecularly dispersed or amorphous drug. However, the detection of a crystalline drug that has phase separated from the nanoparticles is problematic when using the methods commonly employed for the diffractometric investigation of solid lipid nanoparticle dispersions, as demonstrated in measurements on dispersions containing microscopically visible drug crystals [91]. In particular, when comparatively few large drug crystals are formed, the drug particles may sediment out of the beam during measurement or may simply provide too few adequately oriented reflection planes to be detected.

The particle size–dependent line-broadening effect was used to assign the single transitions in complex DSC heating curves of small triglyceride nanoparticles to particle fractions of different, distinct thickness via a sophisticated analysis of the line shape of the corresponding x-ray reflection [19]. In some diffractograms of triglyceride nanoparticle preparations, the occurrence of additional small-angle reflections has been observed, indicating a very large repeating unit. The reflections are caused by the reversible formation of superstructures in the form of stacks of the platelet-shaped particles [92,93]. These examples illustrate that XRD has much broader applicability in the characterization of lipid nanoparticle dispersions than in the simple detection of crystalline material and assignment of crystal polymorphs

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and that XRD can be used to reveal surprising details of the ultrastructure of lipid nanosuspensions, even in a quantitative manner. First approaches to characterize the fine structure of lipid nanoparticle dispersions with neutron scattering have also been reported [92].

3.5 PARTICLE MORPHOLOGY AND ULTRASTRUCTURE OF THE DISPERSIONS

Because of the small size of the structures of interest, electron microscopic techniques in particular have been employed to characterize the overall structure and shape of solid lipid particles. Particles with a spherical shape would offer the highest potential for controlled release and protection of incorporated drugs, as they provide minimum contact with the aqueous environment, as well as the longest diffusion pathways. Compared to particles with any other shape, spherical particles will also require the smallest amount of surface-active agent for stabilization because of their small specific surface area. However, more anisometric particles could have advantages when active agents are to be incorporated into the surfactant layer or adsorbed onto the surface. Particle shape may thus influence drug loading and release characteristics of solid lipid nanoparticles. In addition, with respect to the interpretation of particle sizing data, knowledge about the particle shape is very useful, as anisometric particle shape may affect the results, especially those from PCS.

Any conclusions about the organization of different components within the dispersions should take the ultrastructure of the systems into consideration. The surface-active agents that act as stabilizers for the nanoparticles are often able to form additional colloidal structures, such as vesicles or micelles, by self assembly. In addition to a potential importance in the formation and stability of the dispersions, such structures contain lipophilic domains that may represent alternative compartments for the localization of incorporated drugs. As a consequence, their presence may affect drug incorporation and release.

3.5.1 Transmission Electron Microscopy

Transmission electron microscopy (TEM) can provide valuable information on particle size, shape, and structure, as well as on the presence of different types of colloidal structures within the dispersion. As a complication, however, all electron microscopic techniques applicable for solid lipid nanoparticles require more or less sophisticated specimen preparation procedures that may lead to artifacts. Considerable experience is often necessary to distinguish these artifacts from real structures and to decide whether the structures observed are representative of the sample. Moreover, most TEM techniques can give only a two-dimensional projection of the three-dimensional objects under investigation. Because it may be difficult to conclude the shape of the original object from electron micrographs, additional information derived from complementary characterization methods is often very helpful for the interpretation of electron microscopic data.

Staining techniques employing heavy metal compounds (e.g., uranyl acetate or phosphotungstic acid) as contrast agents have often been used to demonstrate the

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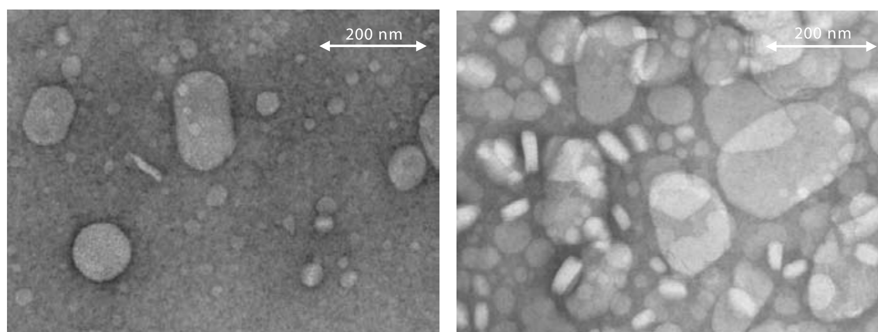

FIGURE 3.4 Transmission electron micrographs of a trimyristin suspension (10% trimyristin stabilized with 4% soybean lecithin and 1% sodium glycocholate) after staining with uranyl acetate solution. In the specimen with lower particle concentration (left), structures that are circular to elongated dominate the image. These structures can also be identified in a higher concentrated specimen (right). The smaller particles of this appearance, in particular, are often of almost circular shape. Because, however, a considerable amount of short, very bright, rodlike structures of a length comparable to the diameter of the circular structures can also be observed, it may be concluded (in good agreement with results from other electron microscopic techniques [Figures 3.5, 3.6]) that at least a fraction of the circular structures corresponds to disks that have adsorbed to the grid with their circular surface. Because of its high phospholipid content, however, the sample may also contain liposomes that cannot clearly be identified in these images.

presence of small particles in dispersions of solid lipids [12,50,52,55,94–99]. Several techniques can be used to place the nanoparticles on the electron microscopic grid (e.g., spraying or passive adsorption from a drop of sample) and to bring them into contact with the staining solution [100]. In any case, the samples have to be dried after staining before they can be viewed microscopically.

Staining techniques have the advantage of being very fast and comparatively simple to use. Moreover, they do not require sophisticated preparation equipment. Unfortunately, the resulting images are often of low resolution, which complicates interpretation. As a further disadvantage, the dispersions do not remain in their original state during preparation because the suspension liquid has to be removed. Drying often leads to artifacts. When preparation relies on diffusion-driven adsorption of the dispersed structures to the surface of the electron microscopic grid, the adsorption process may be selective and the resulting specimen not representative of the whole sample. Anisometric particles (like disks or platelets) may attach in a preferred orientation allowing maximum contact with the grid, and thereby distorting the impression of their real three-dimensional shape (Figure 3.4).

To obtain more detailed information on the ultrastructure of lipid dispersions and the morphology of the particles, electron microscopy is usually performed on replicas of freeze fractured or on frozen hydrated samples. These techniques aim to preserve the liquid-like state of the sample and the organization of the dispersed structures during preparation. By using special devices, the sample is frozen so quickly that all liquid structures, including the dispersion medium, solidify in an amorphous state.

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For freeze fracturing, a very small volume of the dispersion is sandwiched as a thin film between two thin metal holders and rapidly frozen with the aid of a cryogen (e.g., liquid propane or melting nitrogen) [101]. Under cooling, the frozen sample is transferred into a high-vacuum chamber, where it is fractured. The fracture plane is usually not completely smooth, as the sample tends to break preferably at the sites with low binding forces. The remaining relief-like structure may be further elaborated by evaporating a small amount of the frozen water surrounding the particles (freeze etching). The sample surface is shadowed with a carbon/platinum mixture at a certain angle (e.g., 45°), and the resulting thin film is stabilized by the vertical deposition of a thicker film of pure carbon. After the specimen is removed from the freeze fracturing device, all of the original dispersion is removed by rinsing the film with solvents. Only a replica of the surface structure remains, which is viewed in the electron microscope.

Freeze fracture electron micrographs give information not only about the size and shape of the colloidal structures in the sample but also about their internal structure; for example, solid triglyceride nanoparticles frequently appear as sharply edged isometrical structures with distinct internal layers that can be attributed to the molecular ordering of the crystalline matrix lipids. In contrast, emulsion droplets are imaged as circular structures with an unstructured core, reflecting the liquid state of the matrix. Liposomes, which may be formed by excess phospholipid in phospholipid-stabilized dispersions, are often fractured along the lipid area of their bilayer and therefore appear as small bulbs standing out from the overall plane. In the characterization of lipid nanosuspensions, freeze fracture electron micrographs are very helpful in elucidating the shape and structure of the particles (e.g., demonstrating the crystallinity and anisometric, platelet-like shape of different types of solid triglyceride nanoparticles) (Figure 3.5) [1,2,16–20]. Changes in particle structure and morphology caused by polymorphic transitions as well as intraparticulate phase separation between matrix triglyceride and incorporated drug were also visualized with this technique [2,68,90]. Moreover, freeze fracture electron microscopy has been used to investigate the formation of superstructures in colloidal dispersions of solid triglycerides, such as the formation of gels in phospholipid-stabilized dispersions [48] or the stacking of particles in sufficiently concentrated suspensions [92,93]. The morphology and localization of solid paraffin nanoparticles in semisolid preparations has also been investigated by this technique [77].

When concluding on the particle size and shape from images obtained by freeze fracture TEM, the fact that the particles are fractured randomly with respect to the localization of the fracture site within the particle and to orientation toward the fracture plane has to be taken into consideration. Most of the particle remains “hidden” from observation. Therefore, a sufficiently large number of particles have to be investigated to obtain a realistic impression. The determination of a particle size distribution will not be possible when the particles are of anisometric shape.

Cryoelectron microscopy makes it possible to have a direct view into the frozen sample without additional preparation [100]. With the aid of a cryogen (e.g., liquid nitrogen–cooled liquid ethane), the sample is plunge frozen as a very thin aqueous film prepared on a microscopic grid. Subsequently, the vitrified specimen is directly transferred into a precooled electron microscope. Because the specimens are usually

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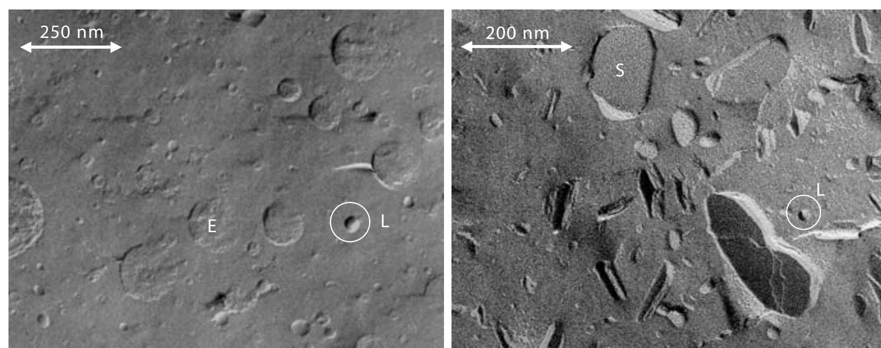

FIGURE 3.5 Transmission electron micrographs of a freeze fractured trimyristin dispersion (10% trimyristin stabilized with 2.4% soybean lecithin and 0.6% sodium glycocholate, prepared by melt-homogenization) stored at room temperature (left) and stored at refrigerator temperature (right). The image to the left has the typical appearance of an emulsion (circular particles with unstructured core [E]) because the matrix lipid does not recrystallize under these conditions. A few liposomes (L) can also be detected. In contrast, the micrograph of the corresponding suspension system (right) displays predominantly anisometric, platelet-like particles with a layered internal structure (S). Liposomal structures are also present in the suspension. (Adapted from [2].)

very sensitive to the electron beam, they have to be viewed under low-dose conditions, avoiding long exposure times. In contrast to freeze fracture electron microscopy, the overall contours of the particles are visible in the plane of projection.

Contrast, which is often quite low for lipid dispersions, depends on the electron optical properties of the structures and on their thickness. As in staining electron microscopy, the three-dimensional particles are projected in a two-dimensional way (e.g., platelet-like crystalline triglyceride particles can be seen as circular to elongated edged particles with low contrast in top-view, or as dark, needle-like structures when viewed edge-on; Figure 3.6). In contrast, spherical emulsion droplets appear as circular, rather dark structures in all orientations [2,3,102]. Unilamellar phospholipid vesicles that may be formed by excess phospholipid can easily be recognized by their ringlike appearance, arising from the phospholipid bilayer [2,48]. Cryoelectron microscopy is thus very well suited to study the coexistence of different colloidal structures such as matrix-type particles and vesicles in a dispersion, as well as to obtain information on the presence of nonspherical particles [2,48,103]. Cryoelectron microscopy has also been used to visualize the presence of different compartments within single nanoparticles in the case of glyceride particles loaded with liquid drug or oil (Figure 3.6) [3,68]. However, it is usually problematic to draw definitive conclusions on the size distribution of the particles under investigation because cryoelectron micrographs tend to be strongly biased toward small particles. This is a result of the preparation technique, which aims at leaving only an extremely thin film on the microscopic grid. Structures distinctly larger than the thickness of this film are either removed during specimen preparation or relocated to thicker film areas (which are usually too sensitive to the electron beam to be reliably investigated)

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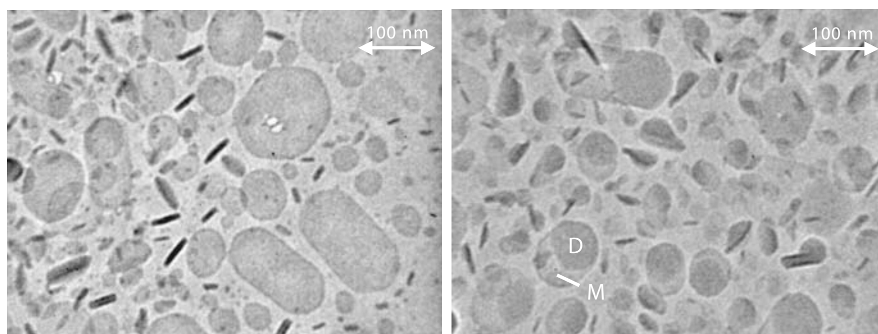

FIGURE 3.6 Cryoelectron micrographs of tripalmitin dispersions (10% lipid phase [triglyceride + drug] stabilized with 2% soybean lecithin and 2% tyloxapol, prepared by melt homogenization) loaded with 10% (left) and 50% (right) ubidecarenone with respect to the dispersed phase. In the dispersion with low drug load (left), the solid lipid particles appear to be circular to elongated, in some cases edged structures with low contrast in top view, and as dark, rodlike structures when projected edge-on. The image also shows indications of size fractionation as a result of differences in film thickness with smaller/thinner structures to the right and larger/thicker, and in some areas superimposed, structures to the left. The particles in the dispersion with high drug load (right) represent two-phase structures: In addition to the structure typical of the crystalline matrix lipid (M), each particle contains a droplet of phase-separated, liquid drug on one of its surfaces (D). These droplets appear as circular areas of higher contrast in top-view and as "cap-like" structures in side-view. (Adapted from [68].)

during this process. Anisometric particles with only one or two large dimensions — such as platelets — may be accommodated by being stretched out parallel to the film surface so that they cannot be viewed edge-on.

3.5.2 Scanning Electron Microscopy

Scanning electron microscopy (SEM) seems to have been used only scarcely for the characterization of solid lipid–based nanoparticles [104]. This method, however, is routinely applied for the morphological investigation of solid lipid microparticles (e.g., to study their shape and surface structure also with respect to alterations in contact with release media) [24,38,39,41,42,80,105]. For investigation, the microparticles are usually dried, and their surface has to be coated with a conductive layer, commonly by sputtering with gold. Unlike TEM, in SEM the specimen is scanned point by point with the electron beam, and secondary electrons that are emitted by the sample surface on irradiation with the electron beam are detected. In this way, a three-dimensional impression of the structures in the sample, or of their surface, respectively, is obtained.

3.5.3 Optical Microscopy

Solid lipid microparticles can also be analyzed by optical microscopy (e.g., with respect to particle size or presence of drug crystals within the particles [41,42,106]),

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whereas the structures in solid lipid nanoparticle dispersions are usually too small to be investigated this way. In spite of this restriction, optical microscopy can be a helpful tool in the study of nanoparticle dispersions, particularly for the detection of phase-separated crystalline drugs [65,81], as well as for the characterization of microparticulate contaminations, as outlined above.

3.5.4 Atomic Force Microscopy

To date, there are only few reports on the use of atomic force microscopy (AFM) for the investigation of the morphology of solid lipid nanoparticle preparations [54,107–109]. The size and shape, in particular, and also the mechanical properties of the sample surface were under investigation in these studies. In contrast to the situation in electron microscopy, information on the three-dimensional extension of single solid particles can be obtained by AFM. Although it is, in principle, possible to investigate hydrated samples with AFM, most research reported so far has been performed on dried specimens. In any case, the particles have to be fixed on a very smooth surface for investigation, usually by adsorption. The sample is investigated by scanning with a very fine probe and monitoring the forces acting on the probe that are caused by interaction with the sample surface. This can be done with the probe either in contact or not in contact with the sample surface (contact of the probe tip with the sample has been reported to cause artifacts because of mechanical interference with the sample).

3.6 INTERACTION WITH INCORPORATED DRUGS

In spite of a rapidly increasing amount of literature data on drug-loaded solid lipid nanoparticle dispersions, there is comparatively little systematic knowledge about the interaction of drugs with these complex systems (e.g., with respect to the state and localization of the drug within the dispersions). One reason may be that the type of interaction is expected to be quite specific for each drug/particle matrix combination, and this interaction may also depend on the general composition of the dispersion and the preparation procedure.

It has to be assumed that the crystalline matrix in solid lipid nanoparticles is a rather unfavorable localization for the incorporation of at least larger amounts of drugs because drug incorporation will disturb the order of the crystal lattice. The incorporation capacity will depend not only on the type of matrix material (e.g., pure triglycerides are assumed to provide a lower incorporation capacity than complex lipids) and its state with respect to degree of crystallinity and polymorphic form, but also on the characteristics of the drug to be incorporated. Some drugs may more favorably interact with the solid matrix than others. Excess drug that cannot be accommodated within the crystalline matrix may adsorb to the surface of the nanoparticles or separate from the particles, either in pure form (e.g., as drug crystals or droplets [64,65,81]) or redistribute to other colloidal structures that may be present in the dispersion (e.g., micelles or phospholipid vesicles) or into the aqueous phase. Many analytical investigations of drug loading of solid lipid nanoparticles were performed on the whole dispersion, often after drying [46,56–58,74,94,97,110–112].

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Investigations such as these yield valuable data on the drug concentration of the overall dispersion but this concentration is the sum of drug incorporated in or adsorbed to the nanoparticles, potentially phase separated drug or drug solubilized in additional colloidal structures, and drug dissolved in the aqueous phase (the latter may be accounted for after separation from the dispersion; e.g., by ultrafiltration). In some cases, additional processing steps, such as washing by centrifugation or ultrafiltration, that are performed before analysis may remove much of the drug present in locations other than in the particles, but the overall value still cannot give information about how much of the drug is actually associated with the nanoparticles or about the type of interaction. Such information may, however, be very important to understand the performance of the dispersions *in vitro* as well as *in vivo*.

The detection of a drug that has phase separated from the nanoparticles may be comparatively easy when large crystals that can be detected by light microscopy are formed [64,65,81], but it could be problematic if the drug does not crystallize or if it forms nanoparticles on its own. Assessing the drug distribution between the aqueous phase and the nanoparticles usually requires a separation of the two phases (e.g., by ultrafiltration, centrifugation, or gel chromatography) [11,36,49,72,96,113,114]. Depending on the separation technique, additional colloidal structures may or may not contribute to the amount of drug detected in the aqueous phase. Indications for the association of incorporated drug with the lipid nanoparticles were, for example, obtained via alterations of the thermal properties of the matrix lipid, such as the melting and recrystallization temperature [52,68,81]. The association of ubidecarenone with triglyceride nanoparticles was also reflected in a change of their polymorphic behavior and their density [68].

Interaction with such properties does, however, not allow the investigator to answer the question of where the drug is localized in the particles (on the surface or in the crystal lattice). Thermal interactions were also observed when an incorporated drug or a second type of triglyceride formed a separate phase within the nanoparticles [3,37,64,68]. Drug release studies can provide supportive information on the accessibility of the drug to the aqueous phase [72,108], but separation of the effects from the nanoparticles from those of additional colloidal structures — if present — may be difficult.

The use of spectroscopic techniques is a very promising approach for evaluating the interaction of drugs with solid lipid nanoparticle dispersions on a molecular level. For example, it was shown by nuclear magnetic resonance (NMR) spectroscopy, using trimyristin dispersions as model systems, that low amounts of the model drugs diazepam, menadione, and ubidecarenone are more strongly immobilized in solid lipid nanoparticle dispersions than in corresponding emulsion systems [65]. This result demonstrated that the drugs under investigation were indeed associated with the nanoparticles. It was, however, not possible to decide whether the drug molecules were incorporated within the crystalline particle matrix or adsorbed onto the particle surface. Similar observations were made on incorporation of triglyceride oil into lipid nanoparticle suspensions [34,37]. In principle, NMR investigations should allow even more detailed insights into the type of interaction of the drug with the nanoparticulate system, but the full potential of this technique still remains to be exploited. As a drawback, highly immobile molecules, as expected on incorporation

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into the solid particles, do not give an analyzable signal in conventional high-resolution NMR spectroscopy. Investigation by magic angle or off–magic angle spinning methods may help overcome this limitation [115].

Electron spin resonance (ESR) spectroscopy, also called electron paramagnetic resonance spectroscopy, has also been used to study the interaction of lipid nanoparticle dispersions with incorporated model compounds. In contrast to NMR spectroscopy, which is able to provide information on conventional drug or excipient molecules via the investigation of signals arising from commonly occurring nuclei such as ^{1}H (in particular, but also, e.g., ^{13}C, ^{31}P, and ^{19}F), an ESR signal can be observed only in substances containing unpaired, paramagnetic electrons. ESR spectroscopy thus requires incorporation of a paramagnetic probe molecule, usually containing a nitroxide radical group, as a spin label. Consequently, the information obtained refers to the molecule carrying this spin label and its local environment, not to a "real" drug-containing system. Experiments using spin probes of different structures as model drugs can, however, provide very valuable basic information on the possible types of interaction of foreign substances with lipid nanoparticle dispersions, for example, with regard to their distribution within the systems and local environment [37,60,116]. Because the position of the signal-giving radical within the carrier molecule can be varied, the local environment of the spin label can be investigated with respect to its localization within the molecules [116]. A simple assay based on the reduction of the nitroxide radical by ascorbic acid contained in the aqueous phase can be used to study the accessibility of a spin-labeled model drug in solid lipid nanoparticle dispersions from the aqueous phase, depending, for example, on the structure and lipophilicity of the spin probe and composition of the particle matrix [37,60,61,116].

3.7 CONCLUSION

A wide variety of techniques has been employed to study the characteristics of solid lipid nanoparticle dispersions. Because of the complexity of these systems, a combination of different characterization techniques is the most promising approach to obtaining a realistic image of the sample properties. Unfortunately, the physicochemical characterization of solid lipid nanoparticle dispersions is often rather limited — particularly in studies closer to application (e.g., *in vivo* investigations). As the characteristics of a dispersion may depend on its exact composition and on the method of preparation, it is often difficult to conclude the behavior of one system from that of another. Even though the knowledge of solid lipid nanoparticle dispersions has increased considerably during recent years, there is still a high demand for more detailed investigations, particularly with respect to the interaction with incorporated drugs, but also concerning the effect of different preparation procedures and dispersion compositions. Because variations may affect the properties of the dispersions in unexpected ways, the introduction of new compositions and preparation procedures should always be accompanied by intensive structural investigations.

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