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Lipid Nanoparticles: Production, Characterization and Stability

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Lipid Nanoparticles: Production, Characterization and Stability

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Preface

Rohan Shah, as a young Ph.D. student at Swinburne University, was intrigued by nanoparticles and what they can do for modern society. As a Graduate of Pharmacy, he was aware of many illnesses which can be cured by modern medicines, but he was aslo accutely aware that those medicines were often ineffecual in the real world because of deficiencies in their delivery system. This prompted Rohan to begin a Ph.D. studying his delivery system of choice—lipid nanoparticles. He soon became aware that there was no single book which gave a good, comprehensive, overview of lipid nanoparticles, their preparation and their characterisation. So, in collaboration with his major supervisors, he set out to write such a book.

The book aims to capture, in the one place, our current knowledge of lipid nanoparticles. It will appeal to all practicioners of science and medicine who have an interest in lipid nanoparticles—from fellow Ph.D. students to chemists working in formulations, to industry partners wanting an overview of lipid nanoparticles, to teachers and lecturers wanting information about lipid nanoparticles, all in one place. The book will also appeal to students, lecturers and researchers with a general interest in colloid science.

The book starts with an overview of lipid nanoparticles, their general structure, type and use. It then goes into more detail about the structure and composition of lipid nanoparticles. How they are made and their formulation complexities are discussed in detail, including the use (by the authors) of microwave technology. Characterisation techniques are introduced, many of which are general and have applicability to many areas of colloid and nanoscience. These include partice size, zeta potential, electron microscopy, crystallinity, polymorphism, thermal characteristics and drug loading capacity. The book gives an overview of these techniques, then detailed information about their application to lipid nanoparticles. Finally, stability is discussed in detail, both in terms of colloidal stability and the stability of drugs encapsulated within the lipid nanoparticles.

Most importantly, each section is thoroughly referenced so that the reader can investigate further those aspects they are most interested in.

The authors are well versed in the fields of colloid science, applied microbiology and formulation science. Mr. Rohan Shah has a degree in pharmacy and

a diploma in biotechnology with publications in the field of phytochemistry and colloid chemistry. The topic of his thesis, developing lipid nanoparticles for drug delivery, is ideally suited to the book. Dr. Daniel Eldridge is an enthusiastic young colloid scientist interested in formulation and with experience in the industrial formulation of liposomes for use in encapsulating skin penetrable medications. His other academic areas of interest primarily include metal adsorption processes and water chemistry. Professor Enzo Palombo has worked as a research microbiologist in the health and university sectors for over 20 years. He combines his research activities on gastrointestinal microbiota, food microbiology, environmental microbiology and bioactive compound discovery with his academic responsibilities in the areas of microbiology, virology, food safety and public health. Professor Ian Harding has over 30 years of experience in industrial colloid science; including waste treatment (he is co-inventor of the patented process of adsorbing colloid flotation for removal of aqueous materials from industrial waste); treatment of PET plastic to enable them to be recycled as a food grade product; minimisation of toxic organic solvents in paint manufacture; the use of biomas as an adsorbent for toxic heavy metals; and more recently the formulation of medicines to treat malaria and the development of biodegradable, thermoreversible polymer scaffolds for use in medicine. Professors Harding and Palombo have previously undertaken several collaborative projects including the formulation of solid lipid nanoparticles (topic of this book), risk assessment of colloidal material in drinking water, investigation of traditional medicinal plants for new drug sources and fungal degradation of industrial (including agricultural) waste.

We hope the book proves useful to other researchers interested in lipid nanoparticles and believe that it is an excellent starting reference for the preparation and characterisation techniques of lipid nanoparticle formulations.

The authors would like to thank several people and organisations without which this book would never have eventuated. These include Swinburne University of Technology for providing many of the materials and the work place for the research involved in microwave formulations; the Department of State Development, Business and Innovation, State Govt., Victoria for provision of a Victoria India Doctoral Scholarship (VIDS), and the Australia India Institute for managing that VIDS; colleagues in the Department of Chemistry and Biotechnology at Swinburne for their support and encouragement; Springer-Verlag, Heidelberg, Germany for encouraging and agreeing to publish this work as a SpringerBrief in Pharmaceutical Sciences and Drug Development, specifically; Dr. Isabel Ullmann, Assistant Editor (Life Sciences/Biomedicine Europe II, Editorial); Dr. Jutta Lindenborn and Ms. Amudha Vijayarajan, Project Coordinators for their assistance in preparation of the manuscript; Mrs. Gomathi Karthikeyan, Production Editor and Mr. Ramasubramaniyan V., Production Coordinator for their assistance in production of the book.

Finally, we would like to thank our family and friends, too numerous to mention by name, for their motivation, enthusiasm and support throughout this project.

Contents

Chapter 1
Introduction

Abstract Highly potent, but poorly water-soluble, drug candidates are common outcomes of lead optimization and other such drug discovery programmes. Poor drug solubility presents several challenges including reduced bioavailability on oral administration, lack of fed-fasted equivalence and abandonment of drug molecules in the early stage of development. Amongst many different strategies for improving drug solubility (e.g. micronization, complexation, prodrug design etc.), development of colloidal carriers has been looked upon as a favourable strategy. A few polymer-based and lipid-based colloidal systems including polymeric nanoparticles, liposomes and emulsions have been well-studied. In this book, the more recent colloidal lipid systems are discussed. Lipid nanoparticles present a viable alternative to other, more traditional, colloidal carriers. These nanoparticles not only have the advantages of most colloidal carriers but also reduce the associated shortcomings. Evolved from parenteral emulsions, lipid nanoparticles are composed of lipid materials that are solid at room and body temperatures, such as triglycerides or fatty acids. Lipid nanoparticle structures can be categorized into four different types: solid lipid nanoparticle (SLN), nanostructured lipid carrier (NLC), lipid drug conjugate (LDC) and polymer-lipid hybrid nanoparticle (PLN). These lipid nanoparticles can encapsulate hydrophilic and lipophilic drugs equally well and may potentially act as the next generation of drug carriers applied in pharmaceuticals. This book focuses on the composition, structure, production, characterization and stability of lipid nanoparticles. Particular emphasis has been given to solid lipid nanoparticles and nanostructured lipid carriers.

Keywords Colloidal carriers · Lipid nanoparticles · Solid lipid nanoparticles · Nanostructured lipid carriers

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1.1 The Rationale of Introducing Colloidal Carriers

Despite great success in the lead optimization programmes, highly lipophilic drug candidates are the major outcomes of such critical drug discovery drives (Porter et al. 2007). Although many of these drug candidates have exceptional in vitro potency, lack of significant solubility in water often presents challenges such as (Merisko-Liversidge and Liversidge 2008):

- Poor bioavailability.
- Variations in bioavailability when in fed or fasted state.
- Use of excipients such as co-solvents to improve water solubility may prove to be harsh.
- Drug precipitation after dosing.
- Patient non-compliance.

In recent years, the proportion of such insoluble drug candidates has been estimated to be roughly 70 % of new drug discoveries. In addition, about 40 % of currently marketed immediate-release oral drugs are practically water-insoluble (Kawabata et al. 2011).

Poor water solubility of drugs limits the development of formulations deliverable by oral and parenteral routes. Formulation strategies that have been reported to overcome solubility issues such include size reduction, complexation and solubilisation in surfactant solutions, pro-drug design, derivatization and modulation of crystallinity (Bikiaris 2011). A more recent and successful strategy to improve solubility is drug encapsulation. Encapsulation of drugs in a colloidal carrier system protects the drug from biological degradation, aids in modulation of release kinetics and modifies its biodistribution (Bunjes and Siekmann 2005).

1.2 Introduction to Colloidal Drug Carriers

The introduction of colloidal systems has led to a revolution in the field of therapeutics over the last couple of decades. It has now been established that the use of colloidal drug carriers in drug delivery not only improves the therapeutic index, but also reduces associated side effects. Colloidal drug carriers have been used to achieve the most optimized therapeutic outcomes (Jaafar-Maalej et al. 2012). There is increasing optimism that colloidal drug carriers will elicit significant and innovative benefits in early diagnosis, prevention and control of disease. A broad variety of colloidal drug carriers have been introduced to address the challenge of solubilisation of poorly soluble drugs (Westesen 2000), and based on the material used in their preparation, they are categorized as polymer-based or lipid-based systems.

Polymeric nanoparticles are colloidal carriers composed of biodegradable macromolecular polymers. Emulsion polymerization is a well-established production

method, but involves the use of toxicologically harmful reactive cross-linkers and carcinogenic monomers. Complete removal of these constituents is a difficult task (Reis et al. 2006). Moreover, slow degradation of the polymer results in its accumulation and may produce toxic metabolites (Bunjes and Siekmann 2005). Polymeric injectable depot formulations such as Lupron® (leuprolide), Nutropin® (recombinant human growth hormone), Sandostatin® (octreotide), Decapeptyl® (triptorelin) and Parlodel® (bromocriptine) are being marketed in spite of these limitations.

Lipid-based colloidal carriers have been introduced to overcome the toxicological issues exhibited by polymeric systems. Prominent research has been carried out on lipidic systems including liposomes, nanoemulsions, micelles, cubosomes and lipid nanoparticles. Liposomes, considered to be the first drug carriers, were described in the early twentieth century. However, it was only in the latter half of the century that they were introduced as drug carriers. Successful development of intravenous liposomal formulations such as Doxyl® and Caelyx® (both liposomal doxorubicin), DaunoXome® (liposomal daunorubicin), AmBisome® (liposomal amphotericin B) and dermal formulation such as "Capture" have demonstrated the usefulness of liposomes as lipidic drug carriers. There are, however, some drawbacks associated with the storage stability of the liposomes. Incorporation of a drug into the phospholipid bilayer can decrease the carrier stability. Additional limitations associated with large scale manufacturing and sterilization after production also make commercialisation of liposomes difficult (de Mendoza et al. 2010).

Parenteral emulsions have been used as calorie sources for decades (Waitzberg et al. 2006). These systems are produced in large quantities and also exhibit long-term stability. Due to these advantages, lipid emulsions are becoming popular as drug carriers. Several drug-loaded colloidal emulsions such as Daizemuls®/Diazepam-Lipuro® (diazepam), Liple® (alprostadil), Diprivan® (propofol), Limethason® (dexamethasone palmitate), Lipo-NSAID®/Ropion® (flurbiprofen axetil) and Etomidat-lipuro® (etomidate) are currently available on the market. Lipid emulsions are, however, not without their shortcomings. Drug molecules that have high mobility in the liquid oil droplet may diffuse out of the droplet, disturbing the stabilizing surfactant film. These effects can cause mechanical or electrochemical instability (such as film rupture, reduction in film elasticity or modification of the zeta potential). This, in turn, can induce coalescence or particle growth (Washington 1996). The high mobility of drug molecules within the emulsion droplets allows for quick equilibration in the aqueous phase, a phenomenon called "drug leakage", and causes rapid release of drugs. This limits the use of lipid emulsions as sustained-release formulations (Magenheim et al. 1993; Washington 1996).

The use of solid lipids was introduced to lower drug mobility observed with liquid lipids. Reduction in mobility inhibits drug leakage and also counteracts drug migration into the emulsifier film. The solid core of the colloidal carrier provides better physicochemical stability. A few of the advantages that the solid lipid imparts to the carrier system are outlined in Table 1.1.

Table 1.1 Advantages of using a solid lipid in the manufacture of colloidal drug carriers

Solid core
Enhanced physicochemical stability of colloidal carrier Enhanced chemical stability of encapsulated drug molecules
Reduced mobility of drug molecules
Enhanced mechanical stability Increase in electrochemical stability due to reduction in diffusing drug molecules (which otherwise decrease stability) Prevention of drug leakage Sustained-release of drugs
Static emulsifier-particle interface
Facilitates surface modification Facilitates drug targeting

1.3 Lipid Nanoparticles: History and Scope

Speiser and co-workers were among the first to use solid lipids in the preparation of nanopellets. They used high sheer homogenizers followed by ultrasonication to produce lipid nanopellets for peroral administration (Speiser 1986). Lipospheres, which are carrier systems similar to nanopellets, were later produced by Domb (1993). The use of lipid nanoparticles as drug carriers has been greatly exploited ever since (e.g. Cutanova Cream nano Repair Q10, NanoLipid Restore CLR, IOPE SuperVital, etc.).

1.4 Lipid Nanoparticles: Types

1.4.1 Solid Lipid Nanoparticle (SLN)

Solid lipid nanoparticles (SLNs) are often referred to as the first generation of lipid nanoparticles. Gasco (1993) and Müller and Lucks (1996), for example, prepared SLNs using different production techniques, and the number of research groups actively involved in SLN production has increased rapidly in subsequent years. SLNs have roused increasing attention in the scientific community as promising drug carriers due to their simplicity and versatility. By definition, they have small particle sizes and, in fact, the smaller the particle size the more likely they are to remain stable, the more likely they are to exhibit targeted responses, and the more likely they are to encapsulate large amounts of drugs (Müller et al. 2002; Wissing et al. 2004). The challenge, with SLN production, is to produce a small particle size but without generating a polydisperse sample.

SLNs are colloidal particles derived from oil-in-water emulsions by replacing liquid lipids with a lipid matrix that is solid at body temperature and stabilized by the use of surfactants. A schematic diagram depicting an SLN is shown in Fig. 1.1a. SLNs potentially emphasise the benefits of colloidal carriers discussed earlier (see Sect. 1.2) whilst reducing the probable shortcomings associated with

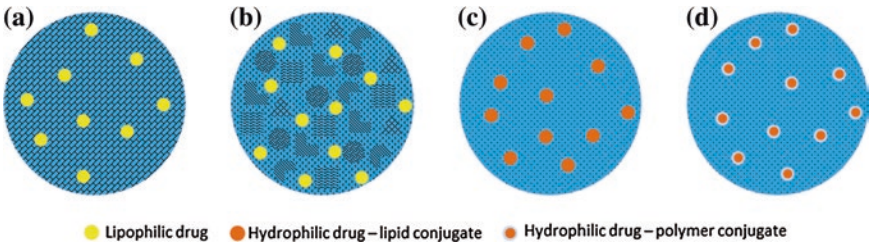

Fig. 1.1 Types of lipid nanoparticles. **a** Solid lipid nanoparticle, **b** Nanostructured lipid carrier, **c** Lipid drug conjugate, **d** Polymer-lipid hybrid nanoparticle

Table 1.2 Potential advantages of solid lipid nanoparticles

Biocompatible and biodegradable colloidal carrier
Prevention of degradation of drug in body fluids due to encapsulation
Increased drug payload
Longer half-life of drug
Possibility of sustained-release and controlled-release of drugs
Longer shelf life
Increased drug dissolution and absorption, improved bioavailability
Drug targeting
Large scale manufacturing
Feasibility of sterilization

them (Jain et al. 2010). Several potential advantages associated with SLNs are listed in Table 1.2 (Das and Chaudhury 2011; Fang et al. 2008; Noack et al. 2012; Saupe et al. 2006).

SLNs have been widely studied for delivery of drugs through dermal (Schäfer-Korting et al. 2007; Souto et al. 2007), peroral (Jenning and Gohla 2001), parenteral (Yang et al. 1999), ocular (Attama et al. 2008; Cavalli et al. 2002; Gasco et al. 2003), pulmonary (Chattopadhyay et al. 2007; Liu et al. 2008; Videira et al. 2002) and rectal (Sznitowska et al. 2001; Sznitowska et al. 2000) routes.

1.4.2 Nanostructured Lipid Carrier (NLC)

Evolved from the SLNs, NLCs were created with a controlled nanostructure of the lipid. NLCs are composed of a binary mixture of a solid lipid and a spatially distinct liquid lipid. As a result, they do not form a perfect crystal. The imperfections present in the solid matrix accommodate the drugs either as molecules or as amorphous crystals (Saupe et al. 2005). A schematic depiction of an NLC is shown in Fig. 1.1b. NLCs have been utilized, for example, in dermal applications (Müller et al. 2007).

The major advantage of NLCs over SLNs is that many drugs are more soluble in a liquid lipid than in a solid lipid. In both cases, the drug is solubilized in the lipid

melt during the initial step of production which is typically performed at a high temperature. The solid lipid then solidifies as the formulation is cooled. During this phase, however, the solubility of the drug can decrease when crystallized into a solid and result in drug expulsion. The use of a liquid lipid in NLC preparation helps reduce drug expulsion during storage (Severino et al. 2012).

1.4.3 Lipid Drug Conjugate (LDC)

Owing to their hydrophobic nature, SLNs and NLCs can only effectively encapsulate hydrophobic drugs. Hydrophilic drugs can only be incorporated at very low concentrations, generally through solubilisation in the lipid melt. Low drug loading means that only highly potent hydrophilic drugs such as proteins and peptides that are effective at very low concentrations are appropriate for use with SLNs or NLCs. Even in these cases, however, low lipophilicity causes difficulty in permeation of such drugs through the gastrointestinal tract (GIT), consequently resulting in poor bioavailability (Muchow et al. 2008). Transforming them into lipid drug conjugates (LDCs) can effectively overcome this situation. The hydrophilic drugs are conjugated with a lipid molecule and transformed into a more lipophilic and insoluble molecule, referred to as "lipid-drug conjugate". Formation of LDCs distinctly reduces drug degradation in the GIT, allowing increased oral absorption and permeation through the GIT (Severino et al. 2012). Figure 1.1c illustrates a schematic depiction of an LDC.

The LDC bulk is often achieved by conjugation of drug to the lipid molecule either by salt formation (with fatty acids) or by covalent linkage such as esterification or amidation. Conjugation by salt formation usually involves solubilizing the free drug base and fatty acid in a suitable solvent. Solvent removal by evaporation is achieved under reduced pressure. In the case of covalent linkage, a reaction between the drug and a fatty acid alcohol in the presence of a suitable catalyst gives an LDC bulk, which is further purified by recrystallization. The recrystallized LDC is homogenized in the presence of a surfactant solution by high pressure homogenization to yield a lipid nanoparticle, specifically an LDC formulation. The LDCs act as prodrugs which are expected to cleave (deconjugation) in vivo to produce the active form of the drug (Müller and Keck 2004). Depending on the partition coefficient of the drug, LDCs can offer improved potential for drug targeting and drug distribution through an organism. Compared to micronized conjugate powder administered orally, LDCs provide enhanced bioavailability.

1.4.4 Polymer-Lipid Hybrid Nanoparticle (PLN)

Polymer-lipid hybrid nanoparticles (PLN) are a variation of SLNs initially developed by Wong and co-workers (Wong et al. 2004). Figure 1.1d shows a structural

depiction of a PLN system. The PLN is a suitable drug carrier for hydrophilic drugs that are usually used clinically in their salt forms. The cationic charges on most of these salts may lead to low drug incorporation into lipid nanoparticles. The use of a counter-ionic polymer such as dextran sulphate to form a drug-polymer complex has been looked upon as an interesting strategy to overcome this. The drug-polymer complex has shown good partitioning into the lipid matrix because of its high hydrophobicity, thereby improving drug incorporation. The PLN system not only allows retarded release but also allows more complete release of some drugs (Wong et al. 2004). These nanoparticles are more attractive because of their capability to encapsulate and deliver multiple drugs (Wong et al. 2006a).

Several researchers have reported increased in vitro anti-tumor activity of doxorubicin-loaded PLNs compared to free doxorubicin solution. The cell lines used in these studies showed increased drug uptake and retention (Wong et al. 2006b, c). The results obtained in vitro could be successfully achieved in vivo in established animal models (Wong et al. 2007).

As the science of lipid nanoparticle technology has progressed, different methods of SLN production have been developed and stable SLN dispersions have been discovered. In this book, the efforts of researchers attempting to incorporate drugs in lipid nanoparticles have been detailed, with the focus on SLNs, and the success that this drug delivery system has achieved to date. Different production methods, characterization techniques and the application of lipid nanoparticles in encapsulation of drugs categorized on the basis of diseases are also discussed.

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Chapter 2
Composition and Structure

Abstract Lipid nanoparticles, including solid lipid nanoparticles (SLN), nanostructured lipid carriers (NLC), lipid-drug conjugates (LDC) and polymer-lipid hybrid nanoparticles (PLN), are colloidal carriers with a lipid matrix that is solid at body temperature. These colloidal carriers have attracted increasing interest for their use in therapeutic and cosmetic applications. The performance of lipid nanoparticle formulations is greatly influenced by their composition and structure. Lipid nanoparticles are generally composed of lipids, surfactants and co-surfactants. The lipid materials used in the production of lipid nanoparticles are usually solid at room temperature. Being well-tolerated in physiological conditions, lipid nanoparticles are typically biocompatible. Liquid lipids, or oils, are specifically used for production of NLCs. In most cases, lipid nanoparticles are produced as dispersions and surface-tailored with surfactants to improve dispersion stability. Polymers are often used to form polymer-lipid cores in the production of PLNs. Lipid nanoparticles are often used as sustained-release systems, with the structure of the lipid nanoparticles dictating their release properties. While the concentration of drug in lipid nanoparticle dispersions is quite well known, knowledge of the drug-lipid interaction in terms of the state and localization of the drug in the nanoparticle is still unknown. Several structural models of SLNs and NLCs have been proposed. The composition and structure of lipid nanoparticles—two critical factors that may influence their pharmaceutical performance—will be discussed in this chapter.

Keywords Composition · Lipids · Surfactants · Localization · Structure

2.1 Composition of Lipid Nanoparticles

SLNs are typically composed of solid lipid(s), surfactant(s), co-surfactant (optional) and active ingredients (typically drugs). The lipids used in the production of lipid nanoparticles are physiological lipids. Based on their structural

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diversity, lipids used in the production are broadly categorized into fatty acids, fatty esters, fatty alcohols, triglycerides or partial glycerides. A few researchers have also reported the use of waxes in the preparation of lipid nanoparticles (Jenning and Gohla 2000). Lipid nanoparticles are surface-tailored with surfactants, which stabilize the colloidal system. They are sometimes used in combination with a co-surfactant, if necessary.

2.1.1 Lipids

The lipid, itself, is the main ingredient of lipid nanoparticles that influence their drug loading capacity, their stability and the sustained release behavior of the formulations. Lipid nanoparticle dispersions based on a variety of lipid materials including fatty acids, glycerides and waxes have been investigated (Blasi et al. 2013a, b; Doktorovova et al. 2014; Durán-Lobato et al. 2013; Dwivedi et al. 2014; Finke et al. 2012; Manjunath et al. 2011; Prombutara et al. 2012; Silva et al. 2011; Wang et al. 2012). Most of these lipids, with the notable exception of cetyl palmitate, are approved as generally-recognised-as-safe (GRAS) and are physiologically well-tolerated.

Selection of appropriate lipids is essential prior to their use in preparation of lipid nanoparticle dispersions. Although there are no specific guidelines, empirical values, such as the solubility of drug in the lipid have been proposed as suitable criteria for selection of an appropriate lipid (Bummer 2004). The solubility of the drug in lipid matrices is critical because it invariably influences the drug encapsulation efficiency and loading capacities, and subsequently the usefulness of the lipid nanoparticles in drug delivery (Kasongo et al. 2011). The solubility of drug can be easily quantified using UV-Visible spectroscopy or chromatographic techniques (Joshi et al. 2008; Joshi and Patravale 2008; Liu et al. 2012). The partitioning of drug between the lipid/oil and aqueous phases can also be predicted using mathematical equations. Such predictions are based on drug-lipid and drug-water interactions. Lipid nanoparticles with high drug loading can be prepared if the drug has high solubility in lipid or a high partition coefficient. Since the drug has different solubility in different lipid matrices, its apparent partition coefficients in those lipids also differ. This consequently leads to different loading capacities in different lipid matrices for the same drug. The complexity thus makes predictive models difficult; however they remain very useful as screening and prediction tools.

Lipid polymorphism is another factor that influences the properties of a lipid nanoparticle system. The occurrence of multiple crystalline forms in solid lipids is particularly useful as they provide structural defects in which drug molecules can be accommodated. The perfect crystalline lattice, however, is more thermodynamically stable than the others. For example, the β-forms of triglycerides are more stable than the α-forms and β′-forms (Chapman 1962). Thermodynamically less stable or metastable forms eventually tend to transform to a more stable form. Such transitions pose a significant challenge in development of SLNs since drug molecules are accommodated in the crystal defects of the solid lipids. Their

disappearance with time thus creates an obvious issue to drug loading. This results in drug expulsion during storage or burst release after administration. Another factor that influences the selection of an appropriate lipid is thus its tendency to form perfect crystalline lattice structures or, at least, the rate at which metastable-to-stable transitions take place. No definitive guidelines exist for the choice of lipids based on these properties.

Generally, crystallisation in lipids with longer chains of fatty acids are slower than those with shorter fatty acid chains (Wong et al. 2007). Wax-based lipid nanoparticles are physically more stable, however they exhibit significant drug expulsion due to their more crystalline nature (Jenning and Gohla 2000). To avoid such problems with lipid crystallinity and polymorphism, a binary mixture of two spatially different solid lipid matrices, i.e. a solid lipid and a liquid lipid (or oil) was used to prepare lipid nanoparticle dispersions, now known as "nanostructured lipid carriers (NLC)" (Jenning et al. 2000d; Müller et al. 2002a; Souto et al. 2004).

Cationic lipids utilised in lipid nanoparticle preparation have been reported for use in gene delivery. The positive charge on the particle surface due to the use of a cationic lipid may enhance transfection efficiencies. Two-tailed (or branched) cationic lipids are preferred over one-tailed cationic lipids due to the cytotoxicity of the latter (Tabatt et al. 2004a, b). Examples of the lipids (including cationic lipids) which have been used in the preparation of lipid nanoparticles, both SLNs and NLCs, are listed in Table 2.1.

2.1.2 Surfactants

Surfactants (also known as surface-active agents or emulsifiers) form the other critical component of the lipid nanoparticle formulation. Surfactants are amphipathic molecules that possess a hydrophilic moiety (polar) and a lipophilic moiety (non-polar), which together form the typical head and the tail of surfactants. At low concentrations, surfactants adsorb onto the surface of a system or interface. They reduce the surface or interfacial free energy and consequently reduce the surface or interfacial tension between the two phases (Corrigan and Healy 2006).

The relative and effective proportions of these two moieties are reflected in their hydrophilic lipophilic balance (HLB) value. Surfactants used in the preparation of lipid nanoparticle preparations play two quite distinct and important roles

- Surfactants disperse the lipid melt in the aqueous phase during the production process
- Surfactants stabilize the lipid nanoparticles in dispersions after cooling

Surfactants can be broadly categorized into three classes based on their charge: ionic, non-ionic and amphoteric. Table 2.2 lists a few surfactants from each class used in the preparation and stabilization of lipid nanoparticles. In all cases, the surfactants are surface tension lowering, which aids in the dispersion process required to form the product (first role). Ionic surfactants are traditionally thought

Table 2.1 Lipids used in the preparation of lipid nanoparticles

Fatty acids	*Waxes*
Dodecanoic acid Myristic acid Palmitic acid Stearic acid	Cetyl palmitate Carnauba wax Beeswax
	Liquid lipids
Monoglycerides	Soya bean oil Oleic acid Medium chain triglycerides (MCT)/caprylic- and capric triglycerides α-tocopherol/Vitamin E Squalene Hydroxyoctacosanylhydroxystearate Isopropyl myristate
Glyceryl monostearate Glyceryl hydroxystearate Glyceryl behenate	
Diglycerides	
Glyceryl palmitostearate Glyceryl dibehenate	
Triglycerides	*Cationic lipids*
Caprylate triglyceride Caprate triglyceride Glyceryl tristearate/Tristearin Glyceryl trilaurate/Trilaurin Glyceryl trimyristate/Trimyristin Glyceryl tripalmitate/Tripalmitin Glyceryl tribehenate/Tribehenin	Stearylamine (SA) Benzalkonium chloride (alkyl dimethyl benzyl ammonium chloride, BA) Cetrimide (tetradecyl trimethyl ammonium bromide, CTAB) Cetyl pyridinium chloride (hexadecyl pyridinium chloride, CPC) Dimethyl dioctadecyl ammonium bromide (DDAB) N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethyl ammonium chloride (DOTAP)

to infer electrostatic stability, whilst non-ionic surfactants are traditionally thought to infer steric repulsion stability. In reality, the situation is much more complex and many non-ionic surfactants used are too small to infer genuine steric stability, but probably result in stability through the Gibbs-Marangoni effect (Walstra 1993).

Members from the Pluronic® and Tween® families are the most commonly used non-ionic surfactants. As discussed, most of these surfactants contain a hydrophilic moiety (ethylene oxide) and a hydrophobic moiety (hydrocarbon chain).

Phospholipids and phosphatidylcholines are the common amphoteric surfactants employed in lipid nanoparticle preparation. These surfactants have both negatively and positively charged functional groups. They exhibit features of a cationic and an anionic surfactant at low and high pH conditions, respectively.

Selection of surfactants for nanoparticle preparation depends on a number of factors, including

- Intended route of administration
- HLB value of surfactant
- Effect on lipid modification and particle size
- Role in in vivo degradation of the lipid

Table 2.2 Surfactants and co-surfactants commonly used in the preparation of lipid nanoparticles

Surfactants	
Ionic surfactants	*Non-ionic surfactants*
Sodium cholate Sodium glycocholate Sodium taurocholate Sodium taurodeoxycholate Sodium oleate Sodium dodecyl sulphate	Tween 20 Tween 80 Span 20 Span 80 Span 85 Tyloxapol
Amphoteric surfactants	Poloxamer 188 Poloxamer 407 Poloxamine 908 Brij78 Tego care 450 Solutol HS15
Egg phosphatidylcholine (Lipoid E PC S) Soy phosphatidylcholine (Lipoid S 100, Lipoid S PC) Hydrogenated egg phosphatidylcholine (Lipoid E PC-3) Hydrogenated soy phosphatidylcholine (Lipoid S PC-3, Phospholipon 80 H, Phospholipon 90 H) Egg phospholipid (Lipoid E 80, Lipoid E 80 S) Soy phospholipid (Lipoid S 75)	*Co-surfactants*
	Butanol
	Butyric acid

Non-ionic surfactants are preferred for oral and parenteral preparations as they are less toxic and exhibit less irritation than ionic surfactants (McClements and Rao 2011). Amongst the ionic surfactants, cationic surfactants are more toxic than anionic or amphoteric surfactants. Therefore, the surfactants arranged in the decreasing order of toxicity are: cationic > anionic > non-ionic > amphoteric. Non-ionic surfactants effectively inhibit the in vivo degradation of lipid matrix. The poly (ethylene oxide) (PEO) chains on the non-ionic surfactants hinder the anchoring of the lipase/co-lipase complex that is responsible for lipid degradation. Adjusting the density of PEO chains on lipid nanoparticle surfaces can modify its in vivo degradation rate. Olbrich et al. studied the effects of surfactants on in vivo lipid degradation (Olbrich et al. 2002; Olbrich and Müller 1999). They suggested that Poloxamer 407 and sodium cholate have the most and least lipid degradation inhibitory effect amongst a selection of tested surfactants.

2.1.3 Other Agents

Apart from lipids and surfactants, lipid nanoparticle formulations can also contain a number of other ingredients including counter-ions and surface modifiers. The lipid nanoparticles engineered for encapsulation of cationic, water-soluble drugs may contain counter-ions such as organic anions or anionic polymers (Cavalli et al. 1995, 2002, 2003).

Table 2.3 Other agents used in the preparation of lipid nanoparticles

Counterions	*Surface modifiers*
Organic salts	Dipalmitoyl-phosphatidyl-ethanolamine conjugated with polyethylene glycol 2000 (DPPE-PEG_{2000}) Distearoyl-phosphatidyl-ethanolamine-*N*-poly(ethylene glycol) 2000 (DSPE-PEG_{2000}) Stearic acid-PEG 2000 (SA-PEG_{2000}) α-methoxy-PEG 2000-carboxylic acid-α-lipoamino acids ($mPEG_{2000}$-C-LAA18) α-methoxy-PEG 5000-carboxylic acid-α-lipoamino acids ($mPEG_{5000}$-C-LAA18)
Mono-octyl phosphate Mono-hexadecyl phosphate Mono-decyl phosphate Sodium hexadecyl phosphate	
Ionic polymers	
Dextran sulphate sodium salt	

Tailoring of the lipid nanoparticle surface with surface-modifiers such as hydrophilic polymers may reduce their uptake by the reticuloendothelial system (RES). The so-called "stealth" or long-circulating carriers stay longer in the systemic circulation and increase the residence of drug in blood (Fundarò et al. 2000; Zara et al. 2002). These "stealth" SLNs have been widely studied for delivery and targeting of anti-cancer cells as they are effectively and selectively taken up by tumor cells (Madan et al. 2013; Pignatello et al. 2013; Priano et al. 2011). Table 2.3 lists some of the counter-ions and surface-modifiers used in lipid nanoparticle preparation.

2.2 Structure of Solid Lipid Nanoparticles

SLNs have three different morphologies, based on the location of the incorporated drug molecule (Fig. 2.1),

- Drug-enriched shell model
- Drug-enriched core model
- Homogenous matrix model

These structures have been described based on the results observed by Müller and co-workers (Müller et al. 2002b).

2.2.1 Drug-Enriched Shell Model

A schematic of the drug-enriched shell model is depicted in Fig. 2.2a. A drug-enriched shell is a lipid core enclosed by a drug-enriched outer shell. Such a structure is obtained when hot liquid droplets cool rapidly to form lipid nanoparticles as a result of phase separation. The drug-enriched shell morphology can

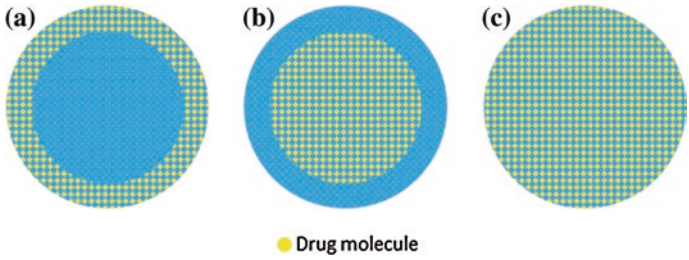

Fig. 2.1 Drug incorporation models of solid lipid nanoparticles. **a** Drug-enriched shell model. **b** Drug-enriched core model. **c** Solid solution (homogenous matrix) model

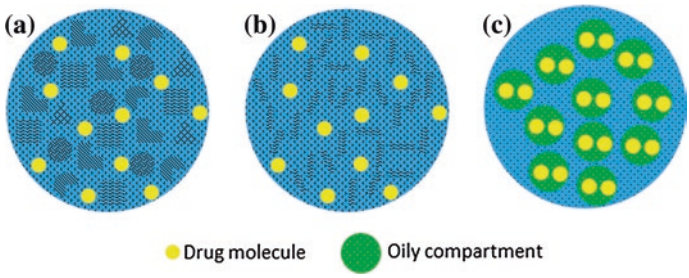

Fig. 2.2 Drug incorporation models of nanostructured lipid carriers. **a** Type I or the imperfect crystal type NLC. **b** Type II or the multiple type NLC. **c** Type III or the amorphous type NLC

be explained by a lipid precipitation mechanism that occurs during production and by repartitioning of the drug that occurs during the cooling stage. After hot homogenization, each droplet is a mixture of melted lipid and drug. Rapid cooling accelerates lipid precipitation at the core with a concomitant increase in drug concentration in the outer liquid lipid. Complete cooling leads to precipitation of a drug-enriched shell. This structural model is suitable for incorporation of drugs that are released as a burst. Such a rapid release is highly desirable for dermatological SLN formulations that require increased drug penetration, in addition to the occlusive effect of the SLN (Muchow et al. 2008). The controlled release of clotrimazole from a topical SLN formulation was due to its drug-enriched shell structure (Souto et al. 2004).

The solubility of the drug in the surfactant-water mixture at elevated temperatures is another factor that can influence precipitation of drug in the shell. During the hot homogenization process, drug partially moves out of the lipid core due to its increased solubility in the surfactant solution. However, solubility of the drug in the surfactant solution decreases as the dispersion is cooled. This leads to drug enrichment in the shell, in cases where lipid core solidification has already started (Muchow et al. 2008).

2.2.2 Drug-Enriched Core Model

A drug-enriched core model is obtained when the recrystallization mechanism is the opposite of that described for the drug-enriched shell model. Figure 2.2b shows a schematic representation of a drug-enriched core model. This morphology is obtained when the drug has a tendency to crystallize prior to the lipid. The drug is solubilized in the lipid melt close to its saturation solubility. Subsequent cooling of the lipid emulsion causes super-saturation of the drug in the lipid melt; this leads to the drug recrystallizing prior to lipid recrystallization. Additional cooling leads to lipid recrystallization that forms a membrane around the already crystallized drug-enriched core. This structural model is suitable for drugs that require prolonged release over a period of time, governed by Fick's law of diffusion (Müller et al. 2002b).

2.2.3 Solid Solution Model

A solid solution model, also referred to as the homogenous matrix model, is obtained when the drug is homogenously dispersed within the lipid matrix in molecules or amorphous clusters. This model is usually described for lipid nanoparticles prepared by a cold homogenization technique, or when highly lipophilic drugs are incorporated such that a hot homogenization technique can be employed without the use of surfactants or drug-solubilizing molecules. When a cold homogenization technique is employed, the solubilized drug is dispersed in the bulk lipid. When subjected to high pressure homogenization, mechanical agitation leads to the formation of lipid nanoparticles with a homogenous matrix. A similar result is obtained when the lipid droplets produced by a hot homogenization technique are rapidly cooled; droplets tend to crystallize and there is no phase separation between the drug and the lipid. Such models are suitable for incorporation of drugs that exhibit prolonged release from particles (Muchow et al. 2008). An example of such a model is a prednisolone-loaded SLN system that exhibits slow release of prednisolone, usually from 1 day to 6 weeks (Jenning and Gohla 2000).

2.3 Structure of Nanostructured Lipid Carriers

Like SLNs, NLCs have been proposed to possess three different morphologies, based on the location of incorporated drug molecules (Jenning et al. 2000a, b, c)

- NLC type I or "imperfect crystal" type
- NLC type II or "multiple" type
- NLC type III or "amorphous" type

2.3.1 NLC Type I or "Imperfect Crystal" Type

Imperfect crystal type NLCs have an imperfectly structured solid matrix. Such imperfections can be increased by using glycerides composed of different fatty acids. Good drug accommodation can be achieved by increasing the number of imperfections. In order to achieve "maximum imperfections", rather than using solid lipids only, the imperfect type of NLC is prepared by mixing spatially different lipids, resulting in imperfections in the crystal lattice. The disordered crystal accommodates more drug molecules, either in molecular form or as amorphous clusters. Using a mixture of glycerides with varying fatty acid chains forms a solid matrix with variable distances. Addition of a small amount of liquid lipid further increases drug-loading (Müller et al. 2002a).

2.3.2 NLC Type II or "Multiple" Type

The second type of NLC is the oil-in-lipid-in-water type. The solubility of lipophilic drugs in liquid lipids (oils) is higher than that in solid lipids. This principle can be used to develop the "multiple" type NLC. In this type of NLC, higher amounts of oil are blended in solid lipids. At low concentrations, oil molecules are easily dispersed into the lipid matrix. Addition of oil in excess of its solubility leads to phase separation producing tiny oily nano-compartments surrounded by the solid lipid matrix. Such models allow controlled drug release and the lipid matrix prevents drug leakage (Jenning et al. 2000d). Lipophilic drugs can be solubilized in the oils and multiple types of NLCs are formed during the cooling process of a hot homogenization process.

2.3.3 NLC Type III or "Amorphous" Type

The phenomenon of crystallization often leads to drug expulsion. To minimise this, NLCs can also be prepared by carefully mixing solid lipids with special lipids such as Hydroxyoctacosanylhydroxystearate, isopropyl palmitate or MCT. Solid, but non-crystalline lipid nanoparticles are formed. The lipid core congeals in an amorphous nature. This type of NLCs, called "amorphous" type NLC, and minimizes drug expulsion by maintaining the polymorphicity of the lipid matrix.

2.4 Conclusions

The physicochemical characteristics and stability of lipid nanoparticles are dependent on the composition of the lipid nanoparticle formulations. The lipid nature of these carrier systems is one of the major features that have attracted the

interest of many researchers. Based on the organisation of lipids and drugs in the particles, a wide variety of structural models have been described for SLNs and NLCs. The drug release from lipid nanoparticles is a compromise between the composition and the structural model obtained for each formulation.

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Chapter 3
Production Techniques

Abstract Besides composition and structure, an important factor which influences the performance and characteristics of lipid nanoparticles is their production method, and a variety of production techniques have been introduced since the inception of lipid nanoparticles as potential colloidal carriers. The production techniques can be categorized into two groups; techniques which require high energy for dispersion of the lipid phase (such as high pressure homogenization, high sheer homogenization, ultrasonication) and techniques which require precipitation of nanoparticles from homogenous systems (such as microemulsions, solvent-based techniques, membrane contactors and coacervation). The choice of an appropriate technique is based on the physicochemical properties of the drug, the physicochemical characteristics and stability of the colloidal formulation and the availability of equipment. This chapter gives an overview of the techniques used in the production of lipid nanoparticle dispersions and also provides a brief introduction to a novel technique based on the use of microwave energy developed by our group.

Keywords Production · Homogenization · Microemulsion · Solvent-based · Coacervation · Membrane contactor · Microwave

3.1 General Considerations

Several approaches for the preparation of lipid nanoparticle dispersions have been reported since these carriers were first described in early 1990s (Gasco 1993; Schwarz et al. 1994; Siekmann and Westesen 1992). The preparation technique has a significant role in the performance of the colloidal formulation. The choice of preparation technique for lipid nanoparticle dispersions may be influenced by:

- Physicochemical properties of the drug to be incorporated
- Stability of the drug to be incorporated
- Desired particle characteristics of the lipid nanoparticle dispersion

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- Stability of the lipid nanoparticle dispersion
- Availability of the production equipment

A brief description of a variety of production techniques is discussed here. Table 3.1 gives a brief outline of the mechanisms involved in lipid nanoparticle formation by various techniques, and the major advantages and disadvantages associated with those techniques.

3.2 Production of Lipid Nanoparticles

3.2.1 High Pressure Homogenization

Being the most reliable and influential technique, high pressure homogenization has emerged as the industry choice of production technique for preparation of lipid nanoparticle dispersions. The method was introduced by Siekmann and Westesen (1992) and Muller et al. (1993), and was later developed and patented for production of SLNs by Müller and Lucks (1996).

Production of SLNs by high pressure homogenization can be achieved by two approaches—the hot homogenization and the cold homogenization approaches.

3.2.1.1 Hot Homogenization

Hot homogenization is a well-established technique from its use in the preparation of fat (or parenteral) emulsions and in dairy processing. Parenteral emulsions are generally produced in temperature controlled cabinets and high temperature is essential for their preparation. Parenteral emulsions are prepared using liquid lipids. Lipid nanoparticles can be drawn from fat emulsions by substituting the liquid lipids (or oils) with solid lipids. Thus, existing production lines for fat emulsions can also be very well engaged in lipid nanoparticle production by the hot homogenization technique (Müller et al. 2000).

Figure 3.1 gives a schematic depiction of the steps involved in the hot homogenization technique. The hot homogenization technique is often regarded as the "homogenization of emulsions" because lipid nanoparticle dispersions are prepared at temperatures above the melting point of the lipid used (Mehnert and Mäder 2001). The solid lipids are melted and the drug is solubilized or dispersed in the molten lipid. The drug-contained lipid melt is dispersed in a hot aqueous surfactant solution, previously maintained at the same temperature, under high speed stirring. Subsequent ultrasonication produces a pre-emulsion. Usually, a piston-gap homogenizer or a jet-stream homogenizer is used to homogenize the pre-emulsion to produce a hot colloidal emulsion. The droplets of the hot colloidal emulsion are recrystallized by cooling the emulsion to room temperature in order to generate SLNs. In some exceptional cases, specific thermal treatment of emulsions such as cooling to refrigeration conditions or even sub-zero temperatures may be required (Bunjes et al. 1996; Lim and Kim 2002; Schwarz and Mehnert 1997; Unruh et al. 2001; Westesen et al. 1997).

Table 3.1 Mechanism, advantages and disadvantages of methods used in preparation of lipid nanoparticles

Production technique	Mechanism of particle formation	Advantages	Disadvantages
High pressure homogenization	High mechanical shear due to strong turbulent eddies Lowering of pressure across the valves of homogenizers Strong cavitation forces	*Hot homogenization* Well established technology Effective dispersion of particles Reproducible High lipid content Simple to scale-up	*Hot homogenization* Extremely high energy inputs (heat and shear forces) High polydispersity Temperature-induced degradation of drugs Complex crystallization; leads to several lipid modifications and occurrence of supercooled melts Inappropriate for hydrophilic drugs; readily distribute in the aqueous phase Reduction in homogenization efficiency at elevated temperatures
		Cold homogenization Effective dispersion of particles Suitable for thermo-sensitive drugs No complex lipid modifications Increased drug-loading due to rapid cooling Suitable for hydrophilic drugs; reduced lipid melting reduces drug loss Simple to scale-up	*Cold homogenization* Extremely high energy inputs Large particles with high polydispersity Drug expulsion on storage
Microemulsion technique	Lipid crystallization due to rapid solidification of microemulsion	Sophisticated equipment not required Low energy inputs Higher temperature gradients; faster lipid crystallization, avoids particle aggregation Simple to scale-up	Low lipid content

(continued)

Table 3.1 (continued)

Production technique	Mechanism of particle formation	Advantages	Disadvantages
Microwave-assisted micro-emulsion technique	Direct coupling of microwaves with molecules Lipid crystallization due to rapid solidification of microemulsion	Controlled microwave heating Rapid and efficient heating Low energy inputs Shorter duration of preparation Higher temperature gradients; faster lipid crystallization	Scalability issues
Solvent evaporation	Lipid crystallization due to solvent evaporation in an anti-solvent	Sophisticated equipment not required Highly suitable for thermo-sensitive drugs Small particle diameters Simple to scale-up	Toxicological issues due to use of organic solvents Particle aggregation in absence of rapid solvent evaporation Low lipid content
Double emulsion	Lipid crystallization due to solidification of emulsion	Sophisticated equipment not required Low energy inputs	Low lipid content
Solvent diffusion	Lipid crystallization due to diffusion of solvent from internal organic phase to external aqueous phase	Sophisticated equipment not required Pharmaceutically accepted organic solvents used; solvent recycling feasible Small particle diameters and low polydispersity Simple to scale-up	Although rare, risk of toxicological risks due to incomplete evaporation of organic solvents Low lipid content
Solvent injection (or displacement)	Lipid crystallization due to rapid diffusion of solvent from internal organic phase to external aqueous phase	Sophisticated equipment not required Pharmaceutically accepted organic solvents used; solvent recycling feasible Highly efficient and versatile technique Higher performance Simple to scale up	Solvent removal difficult; use of freeze-drying or evaporation-under-reduced-pressure Low lipid content

(continued)

Table 3.1 (continued)

Production technique	Mechanism of particle formation	Advantages	Disadvantages
High shear homogenization and/or ultrasonication	Shear between adjacent particles Formation, growth and implosive collapse of bubbles due to cavitation forces	Use of organic solvents can be avoided Use of large amounts of surfactants can be avoided Simple technique with lower production cost Higher energy inputs	Unsuitable for higher lipid contents High polydispersity Physical instability due to high shearing Metal contamination due to ultrasonication Poor encapsulation efficiency
Membrane contactor method	Lipid/oil phase infuses through membrane pores into the tangentially flowing aqueous phase to form droplets Oil droplets crystallize to form lipid nanoparticles	Controlled particle size with selection of membrane with correct pore size Simple to scale-up	Clogging of membrane pores; frequent replacement or cleaning procedures
Supercritical fluid extraction of emulsions	Parallel processes of supercritical fluid extraction (diffusion) of organic solvent from emulsions and lipid dissolution Expansion of organic phase; leads to lipid crystallization	Efficient Rapid and efficient solvent removal Monodispersity Removal of low molecular weight impurities is easy with supercritical fluids Supercritical fluid carbon dioxide causes plasticization of lipid structures; thermodynamically stable lipid nanoparticle dispersions Supercritical fluid lower melting point of lipids; suitable for thermo-sensitive drugs	Use of organic solvents Sophisticated equipment required

(continued)

Table 3.1 (continued)

Production technique	Mechanism of particle formation	Advantages	Disadvantages
Coacervation technique	Decrease in pH of micellar solution of an alkaline salts of fatty acids by acidification (coacervating solution) in presence of a polymeric stabilizer causes proton exchange an lipid precipitation (coacervation)	Suitable for lipophilic drugs (by solubilising in the micellar solution after coacervation) Suitable for hydrophobic ion pairs of hydrophilic drugs Solvent-free technique Use of sophisticated technique not required Monodispersity Simple to scale-up	Suitable for lipids that an form alkaline salts Not suitable for pH-sensitive drugs
Phase inversion temperature technique	Spontaneous inversion of o/w emulsion to w/o emulsion due to thermal treatment (subsequent heating-cooling cycles) Lipid crystallization as a result of emulsion breakage due to irreversible shock induced by rapid cooling	Solvent-free technique Use of large amounts of surfactants can be avoided Combines structural advantages of polymeric nanocapsules and liposomes; imparts stability to the system Suitable for thermo-sensitive drugs Shorter heating periods avoids drug degradation	Particle aggregation Excipients influence the phase inversion behavior Emulsion instability

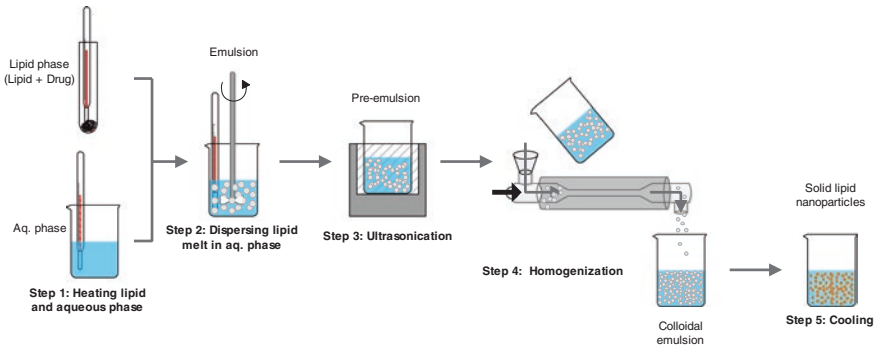

Fig. 3.1 Schematic depiction of steps involved in the hot homogenization technique

The particle size of nanoparticles usually depends on the composition of the dispersions (lipids, surfactants and the dispersion medium) and the homogenization parameters. Particle size can be decreased by increasing the emulsifier-to-lipid ratio, increasing the homogenization pressure, adjusting the homogenization time, increasing the homogenization temperature or adjusting the melt viscosity (Dingler and Gohla 2002; Jenning et al. 2002; Patravale and Ambarkhane 2003; Yang and Zhu 2002). The average particle diameter of SLNs prepared by high pressure homogenization techniques typically ranges from 50 to 400 nm (Blasi et al. 2013a, b; Doktorovova et al. 2014; Durán-Lobato et al. 2013; Dwivedi et al. 2014; Finke et al. 2012; Manjunath et al. 2011; Prombutara et al. 2012; Silva et al. 2011; Wang et al. 2012). Particle size is an important material property that decides the fate of the lipid nanoparticles in the biological system including its elimination from the system (Wu et al. 2011).

3.2.1.2 Cold Homogenization

Cold homogenization techniques are used in the preparation of lipid nanoparticles by passing the predispersed lipid matrix through a high pressure homogenizer at temperatures below the melting point of the lipid. As this technique involves grinding of solid lipids at high pressures, it is sometimes described as "high pressure milling of a lipid suspension" (Mäder and Mehnert 2005).

Figure 3.2 gives a schematic depiction of the cold homogenization technique. Similar to hot homogenization, the solid lipid is heated and drug molecules are incorporated into the matrix by dissolving or dispersing them in molten lipid. The drug-containing lipid melt is rapidly solidified by cooling with dry ice or liquid nitrogen. Rapid cooling favours homogenous distribution of the drug within the lipid material. The solid is then ground into a fine powder by milling into microparticles. The microparticles are subsequently dispersed in a cold aqueous surfactant solution. The dispersion is subjected to high pressure homogenization to generate SLNs.

Cold homogenization involves homogenization of solid lipids as opposed to a lipid melt in hot homogenization. This dispersion of solid lipids requires high

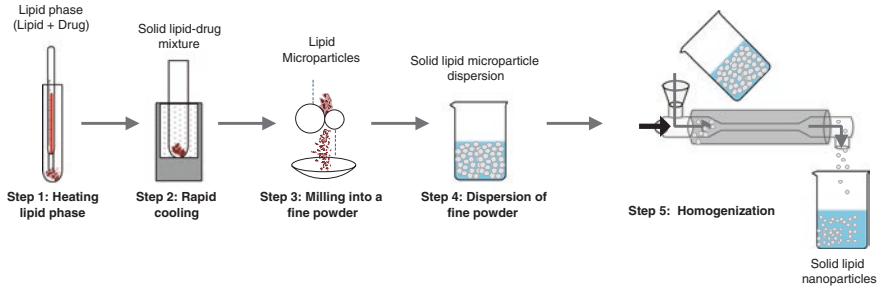

Fig. 3.2 Schematic steps involved in the cold homogenization technique

energy input which in turn requires harsh homogenization conditions. Thus homogenisation itself is more effective for the hot case, and smaller particles which are more monodisperse result (Mäder and Mehnert 2005).

3.2.2 Microemulsion Technique

The preparation of SLN dispersions by precipitation from a hot microemulsion was described by Gasco (1993). A microemulsion is a thermodynamically stable system comprising of water and oil, stabilized by surfactant (and a co-surfactant, if required) and is optically isotropic.

Figure 3.3 depicts the preparation of lipid nanoparticle dispersion by precipitation from a hot microemulsion. The lipid phase and aqueous surfactant/

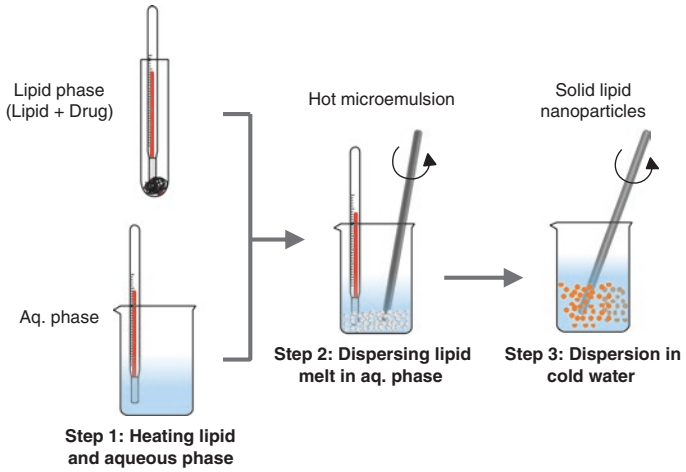

Fig. 3.3 Schematic steps involved in the microemulsion technique

co-surfactant system are separately heated to a temperature above the melting point of the solid lipid. The drug and the lipid are heated together to solubilize the drug in the molten lipid. The lipid melt is later emulsified in the hot surfactant/co-surfactant system under continuous stirring to yield a hot microemulsion, which is then dispersed in cold water (typically 2–4 °C), under mechanical stirring, to yield SLNs. Typically, microemulsion:aqueous phase ratios are 1:25 or 1:50.

3.2.3 Microwave-Assisted Microemulsion Technique

The use of microwave energy in chemical synthesis and processes has been widely investigated in recent years (Gawande et al. 2014). Microwave-assisted synthesis of compound libraries for generation and optimization of new drug candidates is well-established (Hayes 2004). Microwave-assisted drying of pharmaceutical products and long-term stability of solid dispersions of drugs is a widely reported application (Bergese et al. 2003; Moneghini et al. 2008, 2009). Only a few reported successes of the use of microwave energy in pharmaceutical formulation, such as that of polymeric nanoparticles, have been reported (An et al. 2006; Bergese et al. 2003; Waters et al. 2011). The authors of this book have developed a novel production technique for SLNs based on the use of microwave energy (Shah et al. 2014).

Figure 3.4 gives a schematic diagram of steps involved in the microwave-assisted microemulsion technique. The drug, lipid and aqueous surfactant/co-surfactant system are subjected to controlled microwave heating at a temperature above the melting point of the solid lipid. Constant stirring while heating the formulation components in a controlled microwave environment yields a hot microemulsion. Unlike the conventional microemulsion technique, all ingredients are heated in a single synthesis vessel. This step is therefore referred to as "single pot" production of microemulsion. The hot microemulsion obtained from the microwave is again then dispersed in cold water (at 2–4 °C) to generate SLNs.

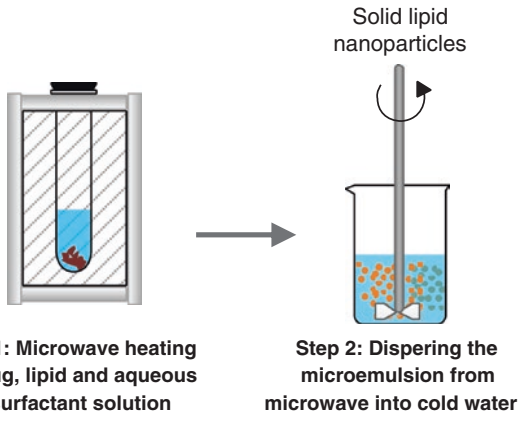

Fig. 3.4 Schematic steps involved in the microwave-assisted microemulsion technique

The use of a controlled environment is the key to successful development of the lipid nanoparticle formulation. The stearic acid lipid nanoparticles prepared by this technique produce particles of approximately 200–250 nm with good physical stability, encapsulation efficiency and drug loading (Shah et al. 2014).

3.2.4 Solvent Evaporation

Solvent evaporation is a well-established technique used in the preparation of pseudolatex (Vanderhoff et al. 1979). The technique has since been used, for example, to prepare lipid nanoparticle dispersions of cholesteryl acetate via precipitation from lecithin-stabilized solvent-in-water emulsions (Sjöström and Bergenståhl 1992).

Figure 3.5 illustrates the solvent evaporation method. The solid lipid is initially dissolved in an organic solvent and the lipophilic drug may also be dissolved in the organic solvent together with the solid lipid. Since their inception, lipid nanoparticle dispersions have been prepared using different organic solvents such as cyclohexane, chloroform and ethyl acetate (Cortesi et al. 2002; Siekmann and Westesen 1996; Sjöström and Bergenståhl 1992). The organic phase is emulsified in an aqueous solution of surfactant to yield an organic solvent-in-water emulsion. A lipid nanoparticle dispersion is then formed on complete evaporation, under reduced pressure, of the organic solvent.

3.2.5 Double Emulsion

The production of lipospheres by the double emulsion method was first described by Cortesi et al. (2002). The double emulsion method was introduced to solubilize

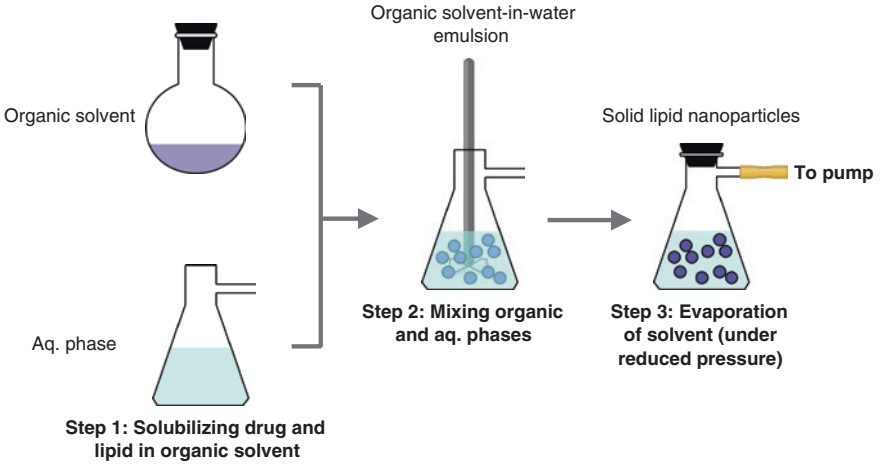

Fig. 3.5 Schematic steps involved in the solvent evaporation technique

hydrophilic drugs in the internal water phase of a w/o/w emulsion, with the aid of a stabilizer to prevent its loss to the external water phase during solvent evaporation.

In a w/o/w double emulsion procedure, an aqueous solution of drug is emulsified in molten lipid to give a primary w/o emulsion and stabilized by adding stabilizers such as gelatin or poloxamer in the aqueous phase. Subsequent dispersion of the primary emulsion in a second aqueous solution of stabilizer under constant stirring generates a w/o/w double emulsion. Constant stirring for longer periods leads to precipitation of lipid nanoparticles. Figure 3.6 illustrates the production of lipid nanoparticles by the double emulsion method.

Garcia-Fuentes et al. (2003) introduced a modified double emulsion technique that was an extension of the solvent evaporation method. The double emulsion was successfully applied to the production of tripalmitin nanoparticles and conveniently modified to prepare surface-coated nanoparticles. Briefly, an aqueous drug solution was emulsified in an organic phase, which had emulsifier and solid lipid previously solubilized into it. The primary emulsion was consequently emulsified in a second aqueous solution to give a double emulsion. The stabilizer in this aqueous solution forms the outer coating of the lipid nanoparticles. The complete removal of solvent was achieved under constant stirring over time. The mean diameter of tripalmitin nanoparticles was 200 nm, using lecithin as the emulsifier. Mean particle sizes of the surface modified nanoparticles ranged from 110–240 nm, depending on the emulsifier in the external water phase. The reproducibility of these results were confirmed by Garcia-Fuentes et al. (2005), who also prepared NLCs by modifying the inner structure with the incorporation of a liquid lipid, Miglyol® 812.

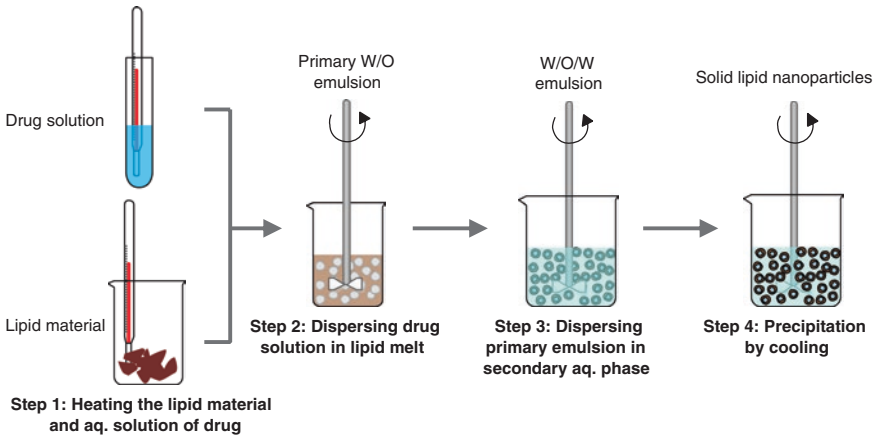

Fig. 3.6 Schematic steps involved in the double emulsion technique

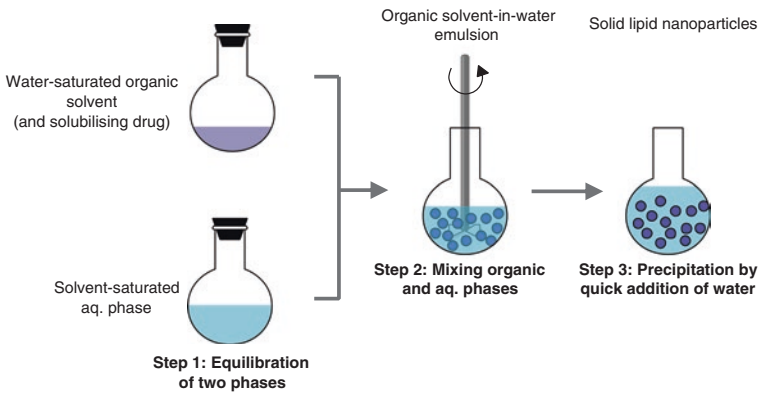

Fig. 3.7 Schematic steps involved in the solvent diffusion technique

3.2.6 Solvent Diffusion

The solvent diffusion method was first introduced in the production of polymeric nanoparticles by Leroux et al. (1995). The method was developed and patented by Quintanar-Guerrero et al. (1996). Hu et al. (2002) introduced a novel solvent diffusion technique in the preparation of lipid nanoparticles.

A schematic protocol for preparation of lipid nanoparticles using the solvent diffusion technique is shown in Fig. 3.7. Partially water-miscible solvents are used to solubilize the solid lipids. A number of solvents partially soluble in water such as benzyl alcohol, butyl lactate, isobutyric acid, isovaleric acid and tetrahydrofuran have been used in the preparation of lipid nanoparticles (Battaglia et al. 2007; Shahgaldian et al. 2003a, b, c; Trotta et al. 2003, 2005). Prior to lipid solubilization, the water-miscible solvents are saturated with water to ensure preliminary thermodynamic equilibrium between the two liquids. The drug may be added to the organic solvent phase. The organic phase is then emulsified with a solvent-saturated aqueous phase containing an emulsifier. Water is added to the primary emulsion to extract the solvent into the external water phase and this generate SLNs. Typical emulsion:water ratios are 1:5 or 1:10.

3.2.7 Solvent Injection (or Displacement)

Solvent injection is a well-established technique from its use in preparation of liposomes and polymeric nanoparticles (Batzri and Korn 1973; Fessi et al. 1989). Solvent injection is a modification of a solvent diffusion technique.

In the solvent injection technique, the lipid is solubilized in a semi-polar water-miscible solvent or water-soluble solvent mixture while the drug is dissolved in the organic phase. The organic phase is rapidly injected, under constant stirring,

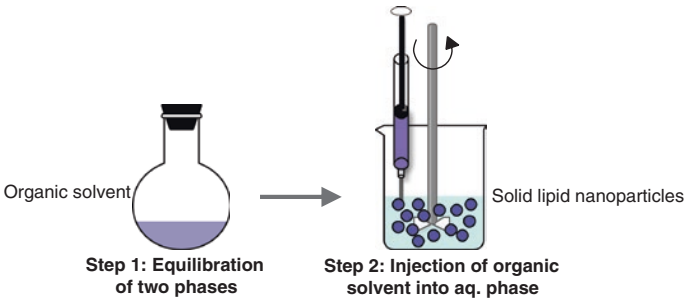

Fig. 3.8 Schematic steps involved in the solvent injection technique

into an aqueous phase containing the surfactant. Lipid nanoparticles precipitate upon solvent distribution into the continuous aqueous phase. A schematic showing the mechanistic steps in solvent injection is depicted in Fig. 3.8.

The particle sizes of the lipid nanoparticles are determined by the velocity of the diffusion of the solvent across the lipid-solvent interface into the aqueous phase. Lipid nanoparticles have been precipitated with polar solvents such as ethanol, acetone, isopropanol and methanol that distribute rapidly into the aqueous phase. Typical diameters of the nanoparticles produced were in the range of 100–200 nm (Dubes et al. 2003; Hu et al. 2002, 2004; Schubert and Müller-Goymann 2003).

Wang et al. (2010) introduced a modified solvent injection method called "solvent injection lyophilization". Lipid nanoparticles were prepared according to the steps discussed earlier for the solvent injection technique except that the organic phase (t-butyl alcohol, in this case) was injected into a stirred aqueous solution containing lyoprotectants to form lipid nanoparticles dispersed in a t-butyl alcohol/water co-solvent system. Subsequent lyophilization of the co-solvent system yielded a dry lipid nanoparticle product which, upon rehydration, formed an aqueous lipid nanoparticle dispersion.

3.2.8 High Shear Homogenization and/or Ultrasound

High shear homogenization and ultrasonication are dispersing techniques. SLN dispersions can be obtained by dispersing a molten lipid in an aqueous phase and then stabilizing with surfactants. Speiser (1986) described the use of high sheer homogenization followed by ultrasonication to prepare lipid nanopellets as an oral drug carrier. The lipid nanopellets obtained had an average particle diameter of 80–800 nm and were suitable for peroral administration.

Lipid nanoparticle dispersions are obtained by dispersing the melted lipid in the warm aqueous phase containing surfactants by high sheer homogenization followed by ultrasonication. Figure 3.9 describes a schematic protocol of manufacturing lipid nanoparticle dispersions by the high sheer homogenization and

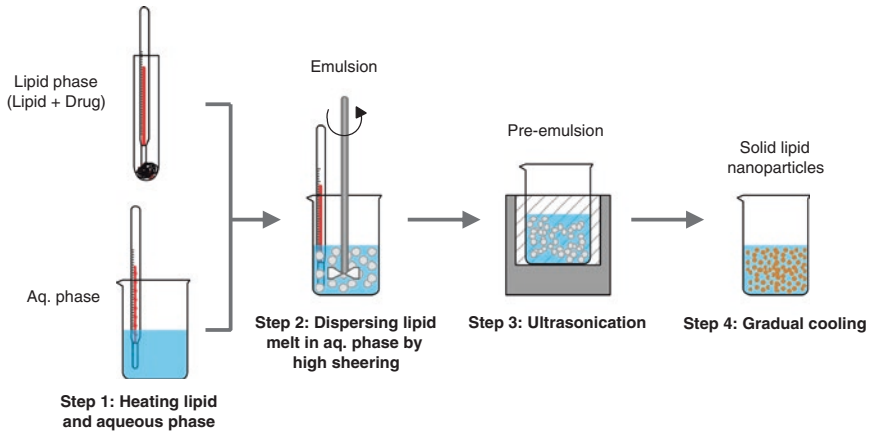

Fig. 3.9 Schematic steps involved in the high shear homogenization and ultrasonication technique

ultrasound technique. This method primarily involves heating of a solid lipid to approximately 5–10 °C above its melting point. The lipid melt is dispersed in an aqueous surfactant solution at the same temperature under high speed stirring to form an emulsion. Subsequent sonication reduces the droplet size of the emulsion. Gradual cooling of the warm emulsion below the crystallization temperature of the lipid yields a lipid nanoparticle dispersion. Concentrated lipid nanoparticle dispersions can be obtained by ultracentrifugation.

3.2.9 Membrane Contactor Method

Membrane contactors have been increasingly used in recent times (Drioli et al. 2003, 2011; Sirkar et al. 1999). Membrane emulsification is a well-established method of preparation of emulsions (Charcosset et al. 2004; Joscelyne and Trägårdh 2000). Membrane contactors have been applied in the manufacture of precipitates including barium sulphate and calcium carbonate (Chen et al. 2004; Jia et al. 2003).

For the preparation of lipid nanoparticles, the lipid melt is initially pressed through the pores of a membrane contactor. The pores in the membrane act as parallel capillaries for introduction of the lipid phase. The passage of the lipid melt allows formation of small droplets into the aqueous phase that flow tangentially to the membrane surface. The aqueous flow carries the droplets formed at the pore outlets to give the lipid nanoparticles. A schematic representation of this method is shown in Fig. 3.10.

The mean particle diameters increase with increasing amount of lipid and the increased lipid content also deteriorates membrane performance, lowering the

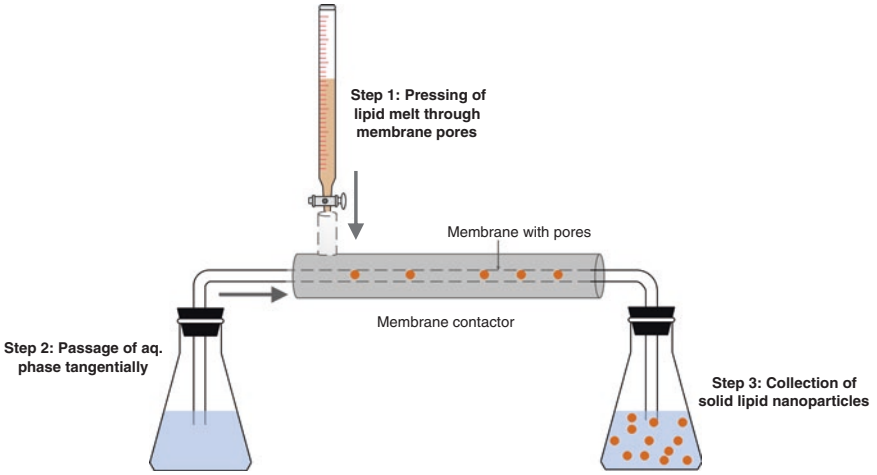

Fig. 3.10 Schematic steps involved in the membrane contactor technique

lipid flux (Charcosset et al. 2005). The dispersed lipid flux is calculated as the volume of the lipid phase divided by the reaction time and membrane surface area. The cross flow velocity and temperature of the aqueous phase also influence the size of the nanoparticles produced and the lipid flux (Charcosset and Fessi 2005). The choice of emulsifiers and their concentration affects the lipid flux and the size of nanoparticles (El-Harati et al. 2006).

3.2.10 Supercritical Fluid Extraction of Emulsions

Supercritical fluid (SCF) technology has been used in the production of microparticles and drugs (Yasuji et al. 2008). SCF technology is based on the principle of precipitation of drug or microparticles using a compressed anti-solvent such as supercritical carbon dioxide. The solutes are dissolved in a solvent. The supercritical fluid chosen here has complete or partial miscibility with the solvent, but acts as the anti-solvent to the solutes. The micron-sized solute particles precipitate upon spraying the solution into flowing SCF (Byrappa et al. 2008).

The process of preparing lipid nanoparticles from emulsions using SCF technology is referred to as "supercritical extraction of emulsions" (SFEE) (Chattopadhyay et al. 2006, 2007). Figure 3.11 is a schematic depiction of the preparation of lipid nanoparticles by the SFEE process. The organic solution is prepared by solubilizing the lipid material and the drug in an organic solvent such as chloroform with the addition of a suitable surfactant. The organic solution is dispersed into an aqueous solution (which may contain a co-surfactant) and the mixture is subsequently passed through a high pressure homogenizer to form an o/w emulsion. The o/w emulsion is introduced from one end of the extraction

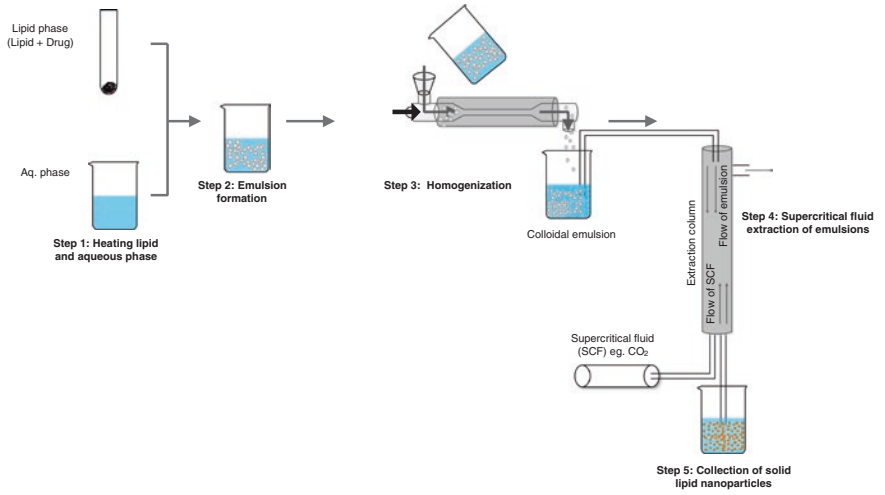

Fig. 3.11 Schematic steps involved in preparation of lipid nanoparticles by the supercritical fluid extraction of emulsions technique

column (usually the top) at a constant flow rate and the supercritical fluid (maintained at constant temperature and pressure) is introduced counter-currently at a constant flow rate. Lipid nanoparticle dispersions are formulated by continuous extraction of solvent from the o/w emulsions.

3.2.11 Coacervation Technique

The coacervation technique has been most commonly employed in the production of polymeric nanoparticles (Maculotti et al. 2009; Silva et al. 2008). Recently, it has been used in the preparation of fatty acid lipid nanoparticle dispersions (Battaglia et al. 2010, 2011). Lipid nanoparticles are produced by acidification of a micellar solution of fatty acid alkaline salts (Bianco et al. 2010; Chirio et al. 2011; Gallarate et al. 2010).

Prior to preparation of lipid nanoparticles, a stock solution of polymeric stabilizer is prepared by heating in hot water. A sodium salt of the fatty acid is homogenously dispersed in the polymeric stabilizer stock solution and the solution is heated above the Krafft point of the sodium salt of the fatty acid, under constant stirring, to obtain a "clear" solution. The drug (solubilized in ethanol) is later added to the clear solution, with constant stirring, until a single phase is obtained. Gradual addition of coacervating solution (or on acidifying the solution) to this mixture yields a suspension. Further cooling of the suspension in a water bath, under constant agitation, yields drug-loaded nanoparticles which are well dispersed. Figure 3.12 gives a schematic depiction of the coacervation technique used in the preparation of lipid nanoparticles.

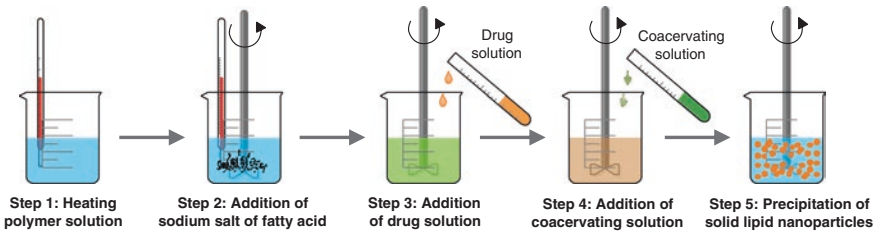

Fig. 3.12 Schematic steps involved in the coacervation technique

3.2.12 Phase Inversion Temperature Technique

Emulsification by the phase inversion temperature (PIT) method was first reported in 1968 by Shinoda and Saito (1968). Transformation of an o/w type to a w/o type of emulsion is termed "phase inversion", can be induced by changing the temperature, and the temperature at which the inversion occurs is referred to as the PIT. Rapid cooling of an emulsion prepared at the PIT produces a fine, stable emulsion. This procedure was used in the preparation of stable lipid nanocapsules in solvent-free conditions by Heurtault et al. (2002). Lipid nanocapsules are an intermediate between lipid and polymeric nanoparticles.

Heurtault et al. (2002) developed a novel solvent-free technique for the formulation of lipid nanocapsules that was based on the phase inversion of an emulsion. In this PIT technique, the formulation ingredients (i.e. lipid, surfactant, drug and water) are thoroughly mixed under constant magnetic agitation. The mixture is then subjected to three cycles of heating and cooling (from room temperature to 85 °C (the PIT), to 60 °C to 85 °C to 60 °C to 85 °C to room temperature) applied at a constant rate of 4 °C/min. In the final step, the emulsion is diluted under cooling conditions. Figure 3.13 gives a schematic representation of steps involved in phase inversion temperature method.

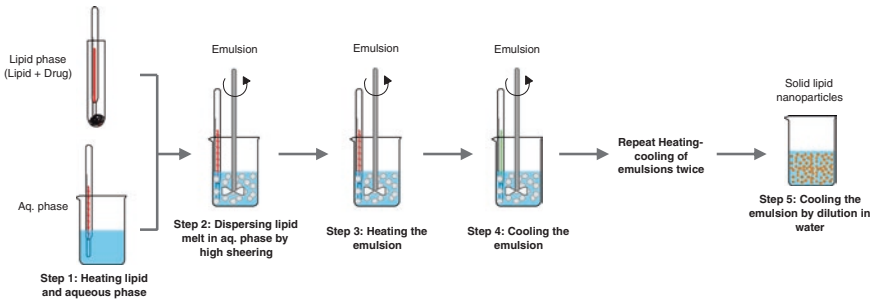

Fig. 3.13 Schematic steps involved in the phase inversion temperature method

3.3 Conclusions

Several production techniques are available and have been discussed in this chapter. The particle characteristics of the lipid nanoparticles is expected to be greatly influenced by the choice of method employed in its production (see next chapter). The choice of method also depends on the composition of the lipid nanoparticle formulation. Table 3.1 outlines the various techniques discussed here with the possible mechanism involved in particle formation. Each of the methods developed to date have their own advantages and disadvantages (also discussed in Table 3.1). A novel method developed by the authors is a rapid technique with many advantages, and few disadvantages, but may face limitations in scalability and is not yet fully tested. Based on literature, it can thus be concluded that no method is perfect, and choice of method will depend on availability of equipment, composition of the lipid formulation and desired property outcomes.

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Chapter 4
Characterization

Abstract Lipid nanoparticles are known to present three features—a solid nature, lipid matrix and colloidal (or smaller) particle size. These features are theorized to impart controlled drug release, biocompatibility and improved drug dissolution to the colloidal carriers. Appropriate characterization of lipid nanoparticle formulations is required to allow for the development of dispersions with the desired properties for the intended application. The structural complexity and the colloidal size of the lipid nanoparticle dispersions make characterization a difficult task. The colloidal size of the particles alters physical features (e.g., increasing solubility and the tendency to form supercooled melts). The properties of bulk materials such as polymorphism and crystallinity may drastically change when these materials are dispersed into nanoparticles. Further complicating characterization is the potential co-existence of other colloidal structures such as micelles, vesicles and emulsions in the dispersions. A detailed characterization scheme is required to investigate the structure and behavior of these complex colloidal carriers. This chapter introduces a number of methods commonly applied for characterization of lipid nanoparticle dispersions. The main focus of the chapter is lipid nanoparticles, however the methods are often generic and occasional mention of their use in other colloidal structures is made.

Keywords Particle characterization · Photon correlation spectroscopy · Laser diffraction · Surface charge · Electron microscopy · Differential scanning microscopy · X-ray diffraction · Nuclear magnetic resonance · Electron spin resonance

4.1 Particle Size

Colloidal particles are defined on the basis of the size of their structure and are defined as having any dimension smaller than 1 μm. Often, colloidal particles are spherical, or at least regular in shape, and particle size measurements can confirm their colloidal size range.

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Particle size can substantially influence the material properties of the nanoparticles. It is a key parameter that decides the fate of nanoparticles in the biological environment. For example, particles greater than 5 μm are detrimental to parenteral administration, since these may cause capillary blockades or embolisms. Particle elimination from the body by the reticuloendothelial system (RES) is also dependent on particle size (Wu et al. 2011).

Formulation parameters (e.g. lipid, surfactant, co-surfactant, dispersing medium and other excipients) and process parameters (e.g. production technique, homogenization time, sonication time, homogenization temperature, homogenization pressure, production equipment, lyophilization and sterilization) are often referred to as principal quality parameters. Each of these factors influences the size and crystallization of particles. The effect of each of these parameters on the particle characteristics, often investigated in terms of particle size, has been reported. An increase in particle size before actual macroscopic changes is often observed in unstable systems. Particle sizing can thus be used as an indicator of formulation instability (Heurtault et al. 2003).

Particle size determination of lipid nanoparticles is often performed by light scattering methods such as photon correlation spectroscopy and/or laser diffraction. The speed and ease-of-use of commercially available equipment has made this technique popular in lipid nanoparticle research.

4.1.1 Photon Correlation Spectroscopy

Photon correlation spectroscopy (PCS), also referred to as dynamic light scattering (DLS), is the most widely used method to ascertain the particle size of lipid nanoparticles in a dispersion (Obeidat et al. 2010). PCS requires a very small amount of sample without extensive sample preparation. It is a rapid, non-invasive and non-destructive technique of sizing colloids. The technique is able to detect particles over the size range of approximately 3–3,000 nm. This operating range is relevant to lipid nanoparticle dispersions. However, this measurement range can be too narrow to detect interference from larger microparticles (>3 μm) (Kaszuba and Connah 2006).

PCS analyzes the random thermal or Brownian motion of the particles suspended in the dispersion medium (Finsy 1994). Particles are irradiated with a laser beam of a particular wavelength. PCS measures the statistical intensity fluctuations in scattered light from multiple collisions of particles as a function of time; collisions arising from random Brownian motion of particles. Small particles, due to their high diffusion coefficient, cause high intensity fluctuations. Large particles, with relatively slower motion, cause lower fluctuations. An autocorrelation function is used to analyze the scattering intensity-time curve to derive parameters that relate to particle size and particle size distribution. The “method of cumulants” can be used to derive the particle size, determined as z-average diameter (also referred to as effective diameter) and polydispersity index (PI), indicative

of the width of the particle size distribution, from the autocorrelation function (Koppel 1972). Particle sizes of lipid nanoparticles are often reported using effective diameter and PI values. These parameters are quite robust and are appropriate for characterizing lipid nanoparticle dispersions. High PI values preclude evocative interpretations of the results based on the method of cumulants. The lower the PI, the more monodisperse the suspension. Most researchers recognize PI values below 0.3 as optimum values; however, values below 0.5 have sometimes been deemed acceptable (Kaur et al. 2008).

Although PCS is the most widely accepted method, it is (like most particle sizing techniques) an indirect method of determination of particle size. PCS is used to characterize particle size depending on its translational diffusion coefficient D. A mathematical model based on the Stokes-Einstein equation (4.1) is used to convert the translational diffusion coefficient into hydrodynamic diameter to determine the particle size,

$$D = \frac{kT}{3\pi\eta d} \tag{4.1}$$

where D is the particle diffusion coefficient, k is the Boltzmann constant, T is the absolute temperature, η is the viscosity of the dispersion medium and d is the diameter of the particle. Particle size determination requires knowledge of the temperature and viscosity of the dispersion medium during measurement.

Although PCS is a simple, robust and reliable technique for characterizing particles having a narrow and monomodal size distribution pattern in nanometer range, it is less useful for dispersions with broad or multimodal size distributions. PCS is not an optimal method when dispersions contain a large portion of particles in the upper-nanometer or micrometer size ranges. The presence of large particles or aggregates may have a significant impact on particle size measurements. The lipid nanoparticle dispersions are often diluted to reduce multiple scattering effects. It should be noted here that dilution of formulations may alter the size distribution, thus resulting the classic problem of changing the measure by the act of measuring it.

Another disadvantage of this technique is the assumption that all particles are spherical. This assumption is less of a concern for SLNs than for other colloidal structures, however it can still be an issue, for example if SLNs crystallize into a platelet-like arrangement (Esposito et al. 2008, 2012). Such anisometric particles exhibit larger hydrodynamic diameter in PCS as compared to corresponding emulsions. Although crystallization leads to volume reduction of particles, anisometric particles have larger particle diffusion coefficients (Westesen et al. 2001). Larger particle diffusions may influence the determination of particle sizes.

PCS has been used by a number of researchers to measure particle size of lipid nanoparticles. Tsai et al. (2012) have reported sizes below 100 nm. Lipid nanoparticles of sizes between 100 and 200 nm have been reported by a few researchers (Gupta and Vyas 2012; Noack et al. 2012; Priano et al. 2011; Varshosaz et al. 2012). Others have reported SLNs with sizes varying from 200 to above 500 nm (de Souza et al. 2012; Jia et al. 2012; Xie et al. 2011; Yang et al. 2013).

4.1.2 Laser Diffraction

Laser diffraction (LD), also called laser light scattering, can be used in combination with PCS. LD is a powerful tool that has a much wider detection range (20 nm–2,000 μm) and is a better choice for lipid nanoparticles in the upper nanometer and micrometer size ranges (Keck and Müller 2008). The two combined are often used to give a complete particle size distribution from ultra-small to large particles.

The operational principle of LD is based on the complex patterns derived due to Fraunhofer, Mie and Rayleigh scattering from an illuminated particle. These patterns are dependent on the particle size of the sample to be analysed. The particle radius is based on the correlation between the angle of diffraction and the particle radius. The light scattered from an illuminated particle is detected by an array of detectors in a laser diffractometer which determines its angular distribution. Large particles predominantly scatter laser light in the forward direction. Smaller particles give a more spherical distribution of scattered light. Thus, the particle size is determined from the geometric distribution of the scattered light. The intensity of the scattered light is also influenced by the particles sizes, and weakens with the cross sectional area of the particle. It can thus be concluded that larger particles scatter light at contracted angles (with higher intensities) as against small particles that scatter light at broader angles (with lower intensities) (Müller et al. 2000).

A laser diffractometer also gives a fair estimation of polydispersity of particles. This technique is well suited for characterization of large microparticles. The Fraunhofer approximation is used to ascertain the diameters of particles in the micrometer and millimetre size ranges. The particle size can be calculated using Mie theory which is a complex combination of optical parameters and angle of scatter. The main drawback of this theory is that its application to nanoparticles requires knowledge of optical parameters (the real and imaginary real refractive indices at the wavelength of measurements) of samples. Particle size distribution is highly influenced by these optical parameters. Optical parameters are not necessary for particles that are 5–6 times larger than the incident wavelength (Keck and Müller 2008).

The use of LD also presents limitations. This technique is less useful in samples containing several populations of variable particle sizes. Uncertainties might develop in instances where particles are non-spherical. Like PCS, an assumption is made that the particles under investigation are spherical. It should also be acknowledged that LD and PCS use light scattering effects to estimate the particle size rather than directly measuring the particle size.

The LD data obtained with an instrument equipped with polarization intensity differential scattering (PIDS) technology has been used to provide more information on particle size distribution (Jores et al. 2004). PIDS technology combines wavelength dependence with polarization effects. This combined approach greatly enhances the sensitivity of LD to smaller particles. However, simultaneous use of PCS and LD for size measurement is recommended. LD has been used by a

number of researchers with who have reported sizes between 100 and 500 nm (Das et al. 2011; Doktorovova et al. 2011; Kovacevic et al. 2011; Mitri et al. 2011; Noack et al. 2012). These researchers have also reported the presence of microparticles in the formulation.

4.1.3 Field-Flow Fractionation

Field-flow fractionation (FFF) is a relatively new technique with the advantage of colloid separation into particle sizes prior to measurement. Particles are separated based on the difference in their Stokes' radii. FFF involves application of electric fields to fluid suspensions or solutions. The fields are applied perpendicular to the direction of flow of the impelling solutions, resulting in separation of particles present in the fluid (Jores et al. 2004). FFF is often more suitable for particle sizing than PCS because of better resolution of small particle size differences. The separation of particles based on their sizes helps in further characterization of the separated particles. Samples characterized by FFF often need to be diluted, so again there are issues with possible changes in particle size on dilution. Dilution of the sample, a major limitation of these method, may lead to changes in concentration of surfactants, salt and other stabilisers thus changing the particle size and/or stability of the particles (Müller et al. 2000).

4.1.4 Other Techniques

Other than light scattering and field-flow fractionation techniques, the Coulter Counter method can also be used for particle size determination. This method uses an electrical zone sensing mechanism to determine the absolute number of microparticles (Freitas and Müller 1998; Schwarz and Mehnert 1997) passing through an aperture of a size which only allows particles of smaller size to pass through. The particle number is determined by measuring electrical resistance which changes when particles pass through the sensing device. The samples have to be diluted in a comparatively concentrated salt solution such as normal saline (0.9 % sodium chloride solutions) prior to measurements. This may interfere with the electrical double layer and subsequently destabilize electrostatically stabilized colloidal particles making the technique unsuitable for a large number of different systems.

Information on particle size may also be obtained from microscopic techniques (Sect. 4.2) such as electron microscopy and atomic force microscopy that are sensitive in the colloidal size range. Unlike light scattering based techniques, the Coulter Counter and Microscope techniques measure individual particles so they have the advantage of a more direct measurement; however they are also quite subjective in real experimentation due to the nature of particle selection. Modern

software imaging devices can help overcome these issues. Single particle counting techniques, such as electron microscopy, also generally make it difficult to observe any aggregation in particles such that only an intrinsic size is obtained and not an effective size.

4.2 Particle Morphology and Ultrastructure

Along with particle size, dispersity and composition, the morphology and ultrastructure are important properties of nanoparticles and their formulations which can control properties such as encapsulation efficiency. Morphology generally relates to the exterior of the particle and may be characterized by shape and surface structure whilst ultrastructure generally relates to the interior of the particle and can relate to internal partitioning through, for example, a core-shell structure. Ultrastructure can also relate to the formulation itself, indicating the presence and orientation of the various components of the formulation. These must include the nanoparticle itself, but can also include structures such as micelles which may be simultaneously present.

Spherical particles have the smallest possible specific surface area for any given shape of particle and hence, are stabilized with the smallest amount of surfactant. Furthermore, because spherical particles have the longest diffusion pathways, they offer the potential for controlled (i.e. slow) release of incorporated drugs. A spherical shape also provides minimum contact with the surrounding aqueous medium, thus, providing protection to the incorporated drugs. On the other hand, anisometric particles require a greater amount of surfactant for stabilization. This is desirable when the drug is to be incorporated into the surfactant layer or adsorbed onto the particle surface. Particle shape, thus, may influence the loading capacity and release properties of the drugs from the lipid nanoparticles. The results obtained from PCS and LD can also be influenced by the anisometric shape of the particles. Particle size results from PCS and LD should therefore be corroborated with electron microscopic techniques to characterize the shape of the lipid nanoparticles.

Different components within the lipid nanoparticle dispersions may organise themselves into a number of different structures, both within and without the nanoparticle itself. The ultrastructure of systems should be given due consideration in predicting the organization of encapsulated drug components within the dispersions. For example, surfactants that stabilize the lipid nanoparticles within the dispersion may self-assemble to form additional colloidal structures such as micelles. Such structures have a lipophilic domain which may pocket some of the drug molecules. The presence of these additional colloidal structures may influence drug incorporation and release. All these factors point out that the performance of lipid nanoparticles—drug incorporation, release and stability are influenced by particle size, shape and structure. It is, thus, necessary to investigate the morphology of lipid nanoparticles.

4.2.1 Transmission Electron Microscopy

Transmission electron microscopy (TEM) is often used to evaluate the morphology and ultrastructure (particle size, shape, structure and presence of other colloidal structures within the dispersion) of colloidal carrier systems (Friedrich et al. 2010). Procedures such as negative staining, freeze-fracture and vitrification by plunge freezing are required for preparation of samples to be evaluated by TEM. Negative staining employing solutions of heavy metal salts is the most commonly used method to demonstrate the presence of colloidal lipid particles in dispersions by TEM (Blasi et al. 2013; Silva et al. 2011). A drop of sample placed on a TEM grid is stained with solutions of heavy metal salts such as uranyl acetate, osmium tetroxide or phosphotungstic acid that act as high contrast agents. Samples are placed on TEM grids by spraying or by passive adsorption and dried before they can be subjected to microscopic imaging.

Staining techniques are quick and easy to use. Sample preparation does not require any special equipment. However, staining and drying of samples may lead to artefacts that are difficult to distinguish from real colloidal structures. The TEM micrographs obtained are of low resolution which interferes with interpretation. For adsorption-related sample preparation, the process depends on diffusion and may be selective. The resulting TEM micrographs may not always be true representations of the entire sample. Most TEM techniques give a two dimensional projection for a three dimensional anisometric particle that may attach to the grid in a preferred orientation (Harris 2007). Figure 4.1 is an example of a TEM image of lipid nanoparticles prepared by the staining technique.

The freeze-fracture technique yields additional information on the ultrastructure of lipid nanoparticle dispersions. A drop of dispersion is placed on a TEM grid which is sandwiched as a thin film between two thin metal holders such as

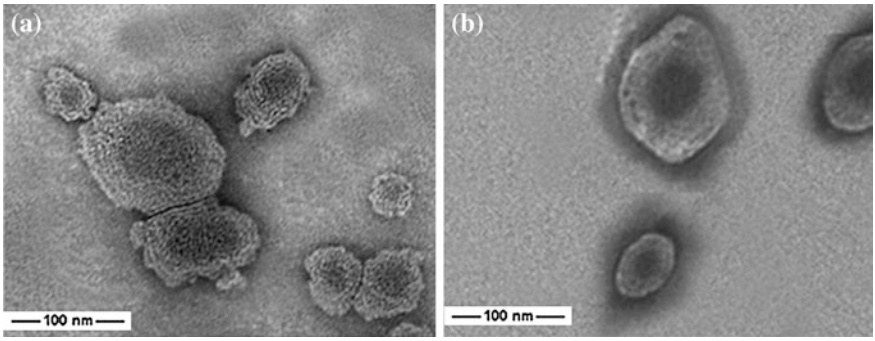

Fig. 4.1 Transmission electron micrographs of nanostructured lipid carriers (**a**) and solid lipid nanoparticles (**b**) after staining with 1 % uranyl acetate solution. (Composition: for NLC: 5 mM glyceryl monostearate, 3 mM egg phosphatidylcholine and 1 mM Pluronic F68, for SLN: 7 mM Stearic acid, 2.5 mM DOTAP, 1 mM Pluronic F68). Reprinted from J Nanopart Res, Jesus et al. (2013), with permission from Springer

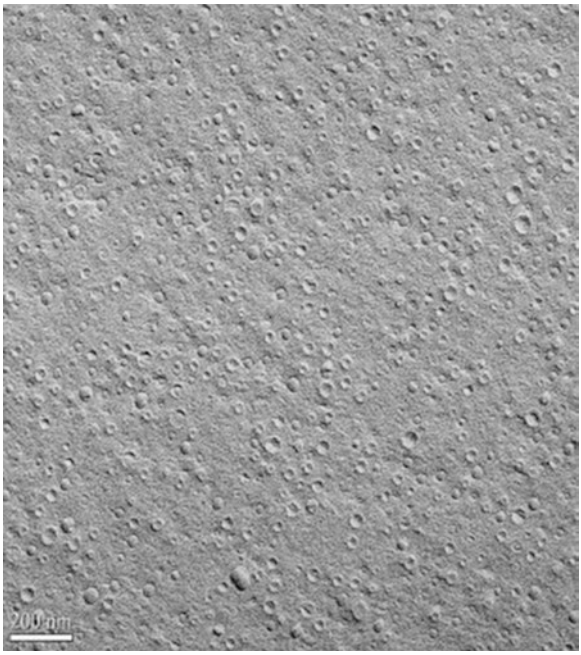

Fig. 4.2 Freeze-fracture TEM of nanostructured lipid carriers prepared by high pressure homogenization. (Composition: 5 % octyl decyl acid triglyceride, 5 % soyabean lecithin, 5 % Coenzyme Q10 and 45 % glycerol). Reprinted from Int J Pharm, Yue et al. (2010), with permission from Elsevier

gold or copper. The drop of dispersion is vitrified by rapid freezing in a cryogen such as liquid propane or liquid nitrogen. The frozen sample is fractured under constant cooling and high-vacuum. The fracture plane develops at the sites with low binding forces. The fracture plane may further be etched by sublimation of the frozen water under vacuum. The sample surface shadowed with a thin carbon/platinum film at a 45° angle provides a negative replica of the fractured plane. The thin film is stabilized with a thicker carbon film (~30 nm). The organic residues are removed by cleaning the replica with an organic solvent and viewing the replica by TEM (Severs 2007). Figure 4.2 is an illustrative example of NLCs prepared by the freeze-fracture technique.

Freeze-fracture transmission electron micrographs are very helpful in elucidating the size, shape and internal structure of colloidal carriers. This technique has been used to distinguish between different colloidal structures such as solid lipid particles, emulsions and liposomes (Westesen et al. 2001). The influence of polymorphic transitions on particle structure and morphology has also been shown with this technique (Bunjes et al. 2003). The freeze-fracture TEM technique was used to visualize the changes in particle morphology due to intraparticulate phase separation between the lipid matrix and incorporated drug molecules (Bunjes et al. 2001).

The freeze-fracture technique has also been employed to investigate the formation of gels in phospholipid-stabilized lipid dispersions, stacking of particles in concentrated suspensions and localization in semi-solid preparations (Unruh et al. 1999). Although freeze-fracture has been widely used in elucidating the

structure of particles, it should be remembered that the particles are fractured randomly, and therefore, a sufficiently large number of particles have to be investigated for a realistic impression of particle morphology, emphasising the point made earlier in this book that number average particle sizing such as microscopy can suffer from subjectivity in particle selection. It is also very difficult to estimate the particle size distribution with this technique particularly because insufficient freezing rates and solvent re-deposition may lead to artefacts.

In contrast to the negative staining and freeze-fracture techniques, cryo-TEM allows direct visualization of vitrified, frozen-hydrated samples without additional preparation. The sample is vitrified in a cryogen as a very thin film on a TEM grid and directly transferred into a pre-cooled microscope for visualization. Cryo-TEM is suitable for investigation of the coexistence of different colloidal carriers such as lipid nanoparticles, vesicles and anisometric particles in the dispersion (Bunjes and Siekmann 2005). Cryo-TEM was a useful tool for understanding the ultrastructure of nanoemulsions, SLNs and NLCs as shown in Fig. 4.3. Localization of oil droplets in the NLCs have been investigated by Cryo-TEM (Esposito et al. 2008; Jores et al. 2004).

In lipid nanoparticle dispersions stabilized with phospholipids, the presence of additional structures such as phospholipid vesicles has been confirmed with cryo-TEM. Cryo-TEM was used to investigate the dependence of particle shape on composition and preparation process (Petersen et al. 2011). Figure 4.4 displays cryo-TEM images of SLNs prepared using trimyristin or cholesteryl myristate and stabilized by different surfactants, and also shows the presence of additional colloidal structures. The major disadvantage of using this technique is that it is strongly biased towards smaller particles and therefore, a definitive conclusion on size distribution cannot be obtained. Structures larger than the thickness of the thin film formed during sample preparation are removed or relocated, leading to unreliable results.

The shape of triglyceride-based lipid nanoparticles was influenced by the type of polymorphic form of triglyceride used in the preparation (Rosenblatt and Bunjes 2008). Figure 4.5 shows an example of TEM images of lipid nanoparticles composed of the same lipid material but different polymorphic modification.

4.2.2 Scanning Electron Microscopy

Scanning electron microscopy (SEM) is the next most commonly used technique for imaging of small particles however it is very rarely used simply to measure particle size of SLNs. It is more commonly used to investigate particle morphology. SEM has been used to investigate the shape and surface structure of SLNs with respect to changes that occur when placed in contact with different release media (Cortesi et al. 2002; Del Curto et al. 2003; Reithmeier et al. 2001a, b; Savolainen et al. 2002). SEM imaging allows observation of SLNs in the absence of aggregation (Dubes et al. 2003). Figure 4.6 displays SEM images of SLNs with

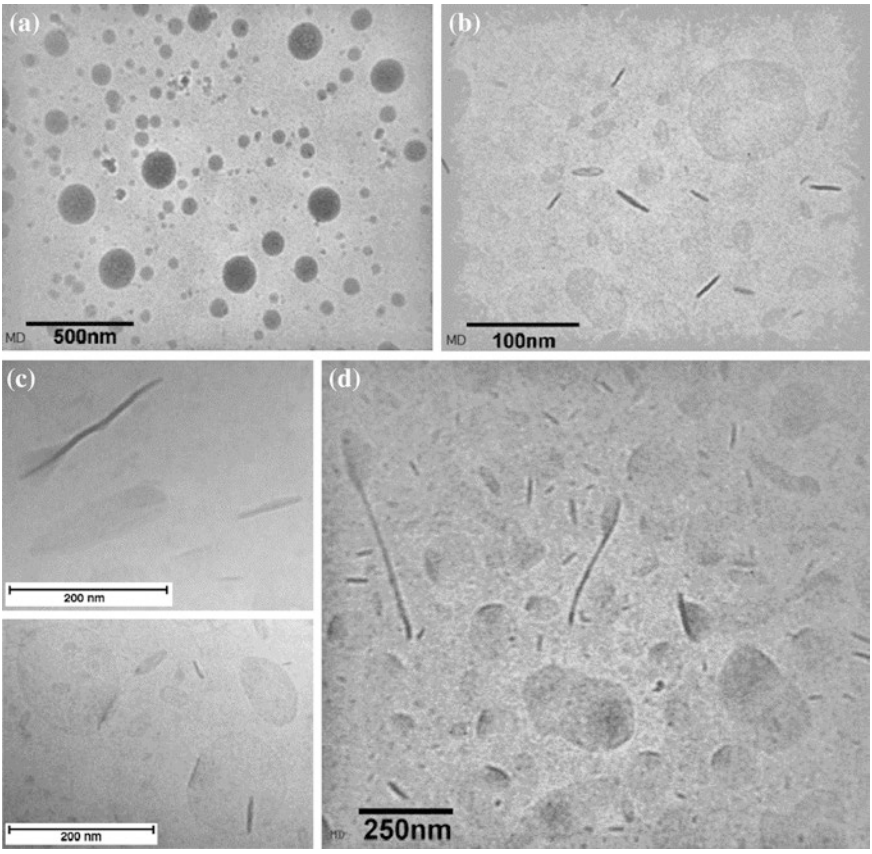

Fig. 4.3 Cryo-TEM images of nanoemulsions (**a**), solid lipid nanoparticles (**b**) and nanostructured lipid carriers (**c** and **d**). (Composition: Nanoemulsion—10 % Miglyol 812; SLN—10 % Compritol 888 ATO; NLC—Compritol 888 ATO: Miglyol (9:1 or 7:3); all stabilized with 10 % Poloxamer 188). Nanoemulsions appeared as droplets, SLN appear as needles (*side view*) or circles (*top view*), NLC appear as platelets or nanospoons (*side view*) or circles with Miglyol sticking to surface (*top view*). Reprinted from J Control Rel, Jores et al. (2004), with permission from Elsevier

or without praziquantel. The particles are usually dried by freeze drying, and their surface is coated with a conducting material such as gold by sputtering.

A major drawback to the technique is that the sample preparation procedures, specifically application of vacuum, may affect the nature of the particles, leading to uncertainty in the experimental observations. This is particularly true in the case of cryo-SEM techniques (as in Fig. 4.6) where freezing must be very quick if artefacts such as salting out of electrolyte during the freezing process are to be avoided.

Cryo-field emission SEM (cryo-FESEM) is another imaging technique used to investigate the ultrastructure of the solid dispersions. Solid particles can be viewed

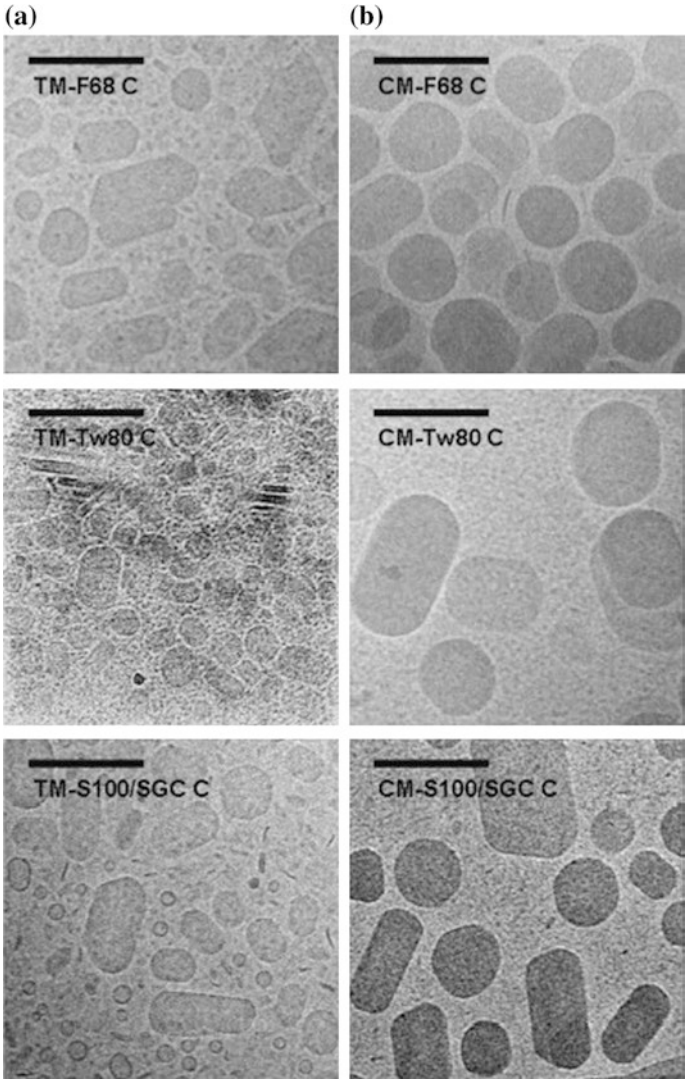

Fig. 4.4 Cryo-TEM images of solid lipid nanoparticles. **a** Trimyristin (*TM*) formulations and **b** cholesteryl myristate (*CM*) formulations, *top row*—Pluronic F68-(F 68) stabilized formulations, *middle row*—Tween 80 (Tw80)-stabilized formulations and *bottom row*—soya bean phospholipid (S100) and sodium glycocholate (*SGC*)-stabilized formulations, *scale bars* represent 200 nm. TM formulations display platelet-like anisometric structures with angular edges and Tw8— and S100/SGC-stabilized lipid platelets appear to have smoother edges than F 68-stabilized lipid platelets. CM formulations display regular lipid platelets. Edges of all platelets, irrespective of stabilizers used, appear to be smooth. Additional colloidal structures such as ring-shaped liposomes and micelles are also seen in S100/SGC-stabilized formulations. Reprinted from Eur J Pharm Biopharm, Petersen et al. (2011), with permission from Elsevier

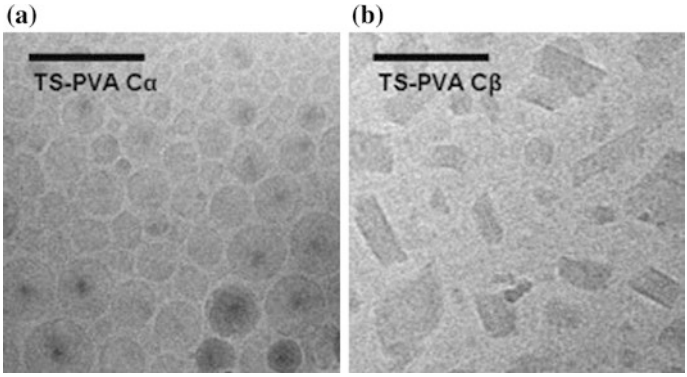

Fig. 4.5 Cryo-TEM images of tristearin nanoparticles stabilized with poly(vinyl) alcohol (*PVA*). **a** Tristearin with α-modification; Cα and **b** tristearin with β-modification, Cβ. Cα particles had a *circular shape* and Cβ particles had *angularly-shaped* platelets. Reprinted from Eur J Pharm Biopharm, Petersen et al. (2011), with permission from Elsevier

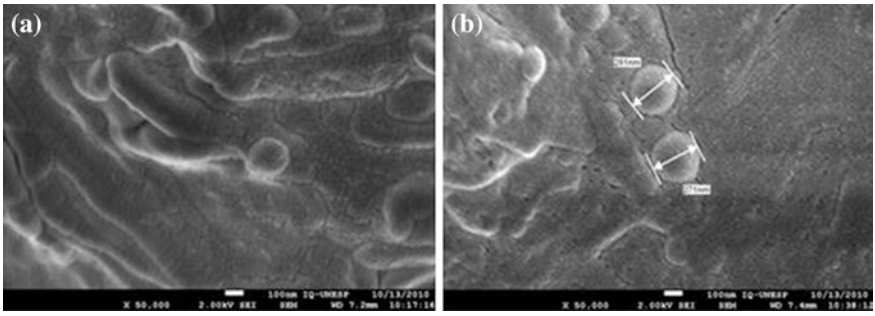

Fig. 4.6 SEM images of unloaded (**a**) and praziquantel-loaded (**b**) solid lipid nanoparticles. (Composition: 5 % stearic acid, 1 % Poloxamer 188). Reprinted from J Therm Anal Calorim, de Souza et al. (2012), with permission from Springer

in their natural state (dispersed in water) by cryo-FESEM (Saupe et al. 2006). Figure 4.7 displays an FESEM image of lipid nanoparticles.

4.2.3 Atomic Force Microscopy

Atomic force microscopy (AFM) has been commonly used to investigate the morphology of lipid nanoparticles (Aji Alex et al. 2011; de Mendoza et al. 2008; Dubes et al. 2003; Olbrich et al. 2001; Shahgaldian et al. 2003; Sitterberg et al. 2010; Tabatt et al. 2004a). AFM provides a high-resolution image of the particle surface and is an important characterization tool for particulate or biological samples as it allows imaging under hydrated conditions. However, probe-sample

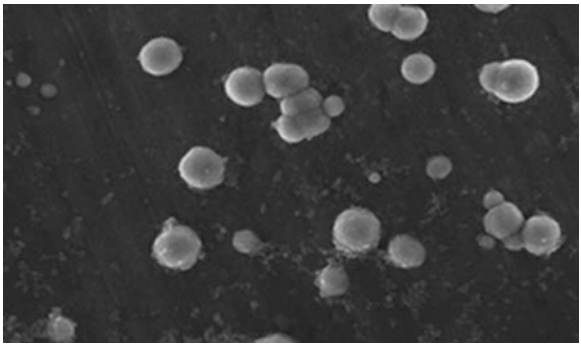

Fig. 4.7 FESEM image of lipid nanoparticles. (Composition: Theobroma oil, oleic acid, beeswax, amphotericin B, sodium cholate and lecithin). Reprinted from AAPS PharmSciTech, Tan and Billa (2014), with permission from Springer

interactions can result in image distortion. In the case of non-contact imaging, a maximum resolution of up to 2 nm can be achieved (Dubes et al. 2003).

Although AFM is suitable for particles in the nanometre to angstrom size range in probe-contact mode, the resolution reduces to as low as 2 nm in non-contact mode. This technique exploits the force acting between the particle surface and the tip of the probe, thereby resolving the image of the particle to as low as 0.01 nm. The major advantage of this particle characterizing technique is that it requires no sophisticated sample preparation. The particle size obtained by AFM is also often close to that obtained by PCS (Dubes et al. 2003) giving confidence in both techniques. AFM has been used to investigate the surface morphology of lipid nanoparticles by a number of researchers (Aji Alex et al. 2011; Chen et al. 2006; de Mendoza et al. 2008; Tabatt et al. 2004a; You et al. 2007).

4.3 Surface Charge

The surface characteristics of colloidal particles have a significant impact on its in vivo behavior and stability. Electrostatic and steric repulsion play an important role in the stabilization of colloidal systems (as discussed in Chap. 5, Sect. 5.2). The surface charge on SLNs is usually due to the presence of ionic surfactants and/or surfactant-dispersion medium interactions, but can also be an intrinsic charge, particularly if the lipid used is an acid.

Traditional inorganic colloidal particles usually acquire their surface charge due to the presence of ionized groups or due to ion adsorption from the medium. Surface charge can be directly measured by acid/base titration in the case of ionized surface groups or can be estimated in the case of adsorbed charge. In either case, however, it is not the surface charge, per se, which is of direct relevance to colloidal behavior. It is the surface potential which results from the surface charge, and perhaps more importantly the potential particles “feel” on close approach which is of importance. Direct measurement of surface potential is, in any case, not possible, however the potential relevant to the distance of close approach during particle interactions is measurable. This potential, the so-called zeta potential,

is both relevant and measureable and therefore serves as the characteristic parameter of choice for nanoparticle charge (Hunter 2002).

The spatial distribution of ions, traditionally referred to as the electrical double layer (EDL) around a charged surface determines its electrical state. The EDL is a physical model consisting of two layers: a fixed layer and a diffuse layer (Fig. 4.8). The fixed layer is a firmly bound layer while the diffuse layer is distributed within the solution in contact with the charged surface. The diffuse layer has an increased concentration of counter-ions. The fixed, bound layer has two surfaces of interest—the genuine particle surface and the surface representing the centre of bound, hydrated counter-ions, often referred to as the "Stern layer". The ions beyond this layer form the diffuse layer, also called the "Gouy" or "Gouy-Chapman layer" (Delgado et al. 2007). The difference in the electrical charge within the EDL results in a potential difference from the surface to the Stern layer to the diffuse layer.

The shear plane is then defined as the plane at which water molecules change from being bound to the surface to being free to move, and is dependent on the energy of mixing (shear). In the simplest case, this is considered a fixed position, as defined in the model presented in Fig. 4.7. The potential at this plane (the "Stern potential") is either equal to, or slightly higher (in magnitude) than the measured potential at the shear plane which, in turn, is referred to as the zeta potential (ζ).

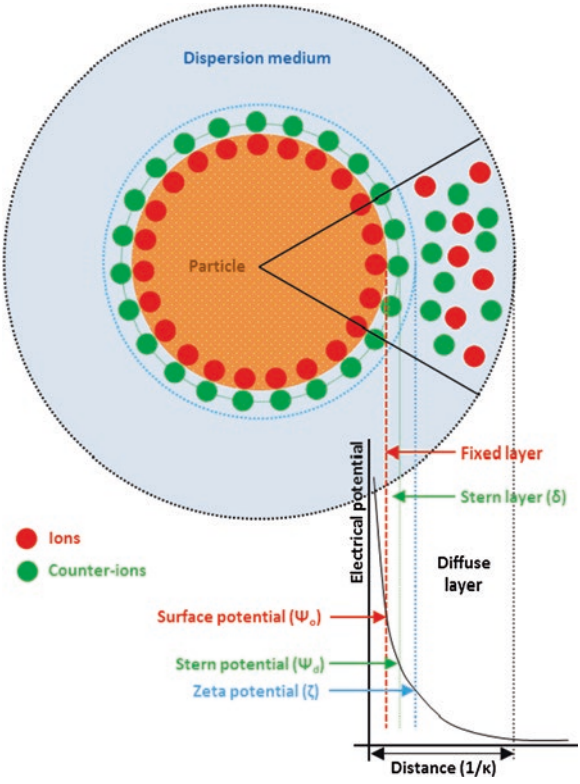

Fig. 4.8 Schematic representation of electrical double layer

The zeta potential is commonly determined by laser Doppler anemometry based on the principle of Doppler shift. A dilute suspension of nanoparticles is subjected to a weak electric field. The light scattered due to moving particles causes a frequency shift which is used to determine the electrophoretic mobility (μ, particle velocity/strength of electric field). The zeta potential is commonly derived from electrophoretic mobility using the Helmholtz-Smoluchowski equation (4.2),

$$\mu = \frac{\varepsilon \zeta}{\eta} \tag{4.2}$$

where ε is the permittivity and η is the viscosity of the dispersion medium. The Helmholtz-Smoluchowski equation is strictly only applicable under conditions of large particle size and/or high ionic strength and these conditions are rarely encountered in nanoparticle research, a common error, therefore, in zeta potential experiments. More sophisticated models include the Henry equation and the Wiersema approach and these are more applicable, but less often used, to nanoparticles. For this reason, zeta potential measurements of nanoparticle formulations should be treated qualitatively rather than quantitatively (see Chap. 5, Sect. 5.2).

The zeta potential is influenced by the pH, ionic strength and the types of ions in the dispersion medium. Similar to particle size determinations using light scattering, it is generally required that zeta potential measurements are conducted on diluted samples to avoid multiple scattering effects (Xu 2008). Measurement of zeta potential of particles dispersed in distilled water or water with very low conductivity provides information about particle surface charge (Radomska-Soukharev 2007).

Usually, lipid nanoparticles carry a negative charge developed by the surfactant system used in its stabilization. However, cationic lipid nanoparticles have also been prepared which find application in DNA and gene delivery (Choi et al. 2008; Doktorovova et al. 2011; Olbrich et al. 2001; Tabatt et al. 2004b).

Zeta potential measurements have been undertaken to study the effect of electrolyte and pH on the stability of SLNs (Choi et al. 2014). The zeta potential of SLNs can be increased by addition of surfactants such as egg phosphatidylcholine or Tween 80 to the surfactant mixture (Lim and Kim 2002). The presence of a cosolvent in the formulation may increase the zeta potential of the system (Trotta et al. 2003). Sterilization and freeze drying of SLNs can reduce the zeta potential. The presence of cryoprotectant can also influence the zeta potential of SLNs (Cavalli et al. 1997; Schwarz and Mehnert 1997; Soares et al. 2013; Varshosaz et al. 2012). Formulation parameters such as surfactants and the lipid matrix also affect zeta potential determinations (Kovacevic et al. 2014, 2011).

4.4 Crystallinity and Polymorphism

The release properties of lipid nanoparticles essentially rely upon the solid state of the particles. Most of the production techniques for lipid nanoparticles require heating of the lipid matrix above the melting point and thus form from a hot

microemulsion which solidifies after dispersion in the aqueous phase. However, many lipid materials may not crystallize in a colloidally dispersed state. The lipid materials may crystallize at a temperature much lower than their melting points. In such cases, the dispersions are regarded as "emulsions of supercooled melts", and not lipid nanoparticle dispersions. Recrystallization of nanoparticles made of short chain triglycerides such as tricaprin, trilaurin and trimyristin requires careful evaluation. Lipid nanoparticle dispersions of glycerides such as Witepsol® and Softisan® usually exhibit retarded crystallization (Choi et al. 2008; Kuntsche and Mäder 2010; Lim and Kim 2002).

Since the modified release properties of lipid nanoparticles are significantly influenced by the crystalline nature of lipid nanoparticles, it is important to study the crystallinity of lipid nanoparticles prepared with novel compositions or techniques. Crystalline solid particles are obtained when colloidal emulsion droplets are cooled below the lipid's critical crystallization temperature. Dispersions of such lipid materials may remain in the emulsion state, and may not form the desired solid state. Special thermal treatment may be required to ensure that particles are formed in the solid state. The critical crystallization temperature is lipid-specific and may be influenced by other components present in dispersion. Bunjes et al. (2002) suggested induced crystallization of triglycerides at higher temperatures due to its interactions with the stabilizer. The presence of partial glycerides and/or residues of long fatty acids in glycerides may reduce the supercooling tendency (Ali et al. 2010; Bunjes et al. 1996; Westesen et al. 1997).

The crystallization tendency of nanoparticles can be further suppressed by the incorporation of drugs. Incorporation of ubidecarenone into tripalmitin-based nanoparticles, for example, reduced the crystallization temperature by 10 °C (Bunjes et al. 2001). The drug molecules are tightly bound to the carrier matrix at low concentrations, but when present in excess, they adhere to the carrier surface as a liquid phase, thus lowering the crystallinity (Bunjes et al. 2001). Addition of liquid lipid leads to suppression of crystallization temperature in NLCs (Ali et al. 2010; Awad et al. 2009; Jenning et al. 2000c). Incorporation of a drug and/or inclusion of liquid lipids also lead to a decreased melting point, however, the reduced melting temperature is usually lower than the crystallization temperature (Müller et al. 2008).

Further changes occur after the initial solidification of SLNs. The solid lipids employed in these production procedures usually display polymorphism which are also displayed in the colloidally dispersed state. In addition to other crystal aging phenomena, polymorphic transitions can occur for several weeks after solidification of nanoparticles (Bunjes and Koch 2005).

The triglycerides occur in three polymorphic forms, the least stable α-form, the metastable β′-form and the most stable β-form. Triglyceride-based nanoparticles usually crystallize in the α-form when solidified from a lipid melt. An intermediate β_i-form of triglyceride nanoparticles has been reported when nanoparticles are produced by hot homogenization. Alterations in matrix components, incorporation of drug molecules and the presence of surfactant molecules can all influence the polymorphic transitions (Bunjes et al. 1996, 2001; Jenning et al. 2000b; Jores et al. 2003; Schubert et al. 2005; Westesen et al. 1997).

These nanoparticles usually transform into more stable forms on storage and on heating, which may not always be desirable. Polymorphic transitions are usually accompanied by changes in shape of the nanoparticles. Alterations in particle characteristics can compromise pharmaceutically relevant properties such as drug incorporation and stability, for example by expelling the drug (on storage) as the nanoparticles become more crystalline, thus lowering the shelf life of the product. These observations suggest that investigation of crystallinity and polymorphic behavior of lipid nanoparticles should be included in the characterization processes.

Differential scanning calorimetry (DSC) and X-ray diffraction (XRD) are the two most widely employed techniques for characterization of crystallinity and polymorphism of SLNs. DSC is a very sensitive technique used in the detection of crystalline materials. The polymorphic form can be detected only indirectly by careful investigation of transition temperatures and melting enthalpies. For such cases, XRD is a more reliable technique which provides more detailed structural data. DSC data can be used to assign polymorphic form when supported by XRD data (Bunjes and Siekmann 2005). An example of such a correlation can be seen in Fig. 4.9.

Preferably, the lipid nanoparticle dispersions should be investigated in their native, dispersed state unless the final formulation is to be marketed as a dry powder for injections, and modern DSC and XRD equipment have the ability to do this. If sample preparation such as diluting the sample or freeze-drying is unavoidable, it should be remembered that such procedures may change particle characteristics such as particle size, crystallinity and polymorphism. Freeze-drying, for example, can lead to phase transitions and changes in particle size. In some cases, when such treatments are unavoidable, dried samples should be checked for the

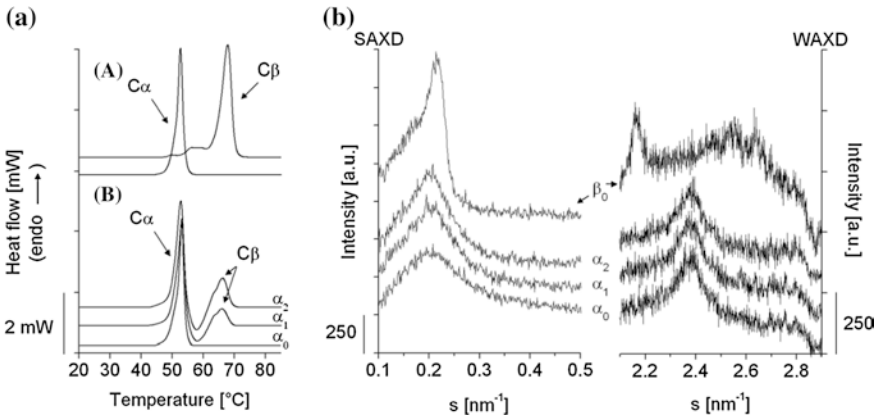

Fig. 4.9 DSC curves and SAXS/WAXS diffractograms of tristearin nanoparticles stabilized with poly(vinyl) alcohol (*PVA*). For DSC curves, *A*—DSC heating curves for lipids in the crystalline form and *B*—DSC curves after incubation at 37 °C (α_0 at time = 0 h, α_1 at time = 1 h, α_2 at time = 2 h). Similarly for SAXS/WAXS diffractograms, β_0 crystalline lipid in β-modification at time = 0 h, α_0 crystalline lipid in α-modification at time = 0 h, α_1 at time = 1 h, α_2 at time = 2 h. Reprinted from Eur J Pharm Biopharm, Petersen et al. (2011), with permission from Elsevier

presence of drug crystals by DSC and potential sample alterations should be considered while interpreting the data (Cavalli et al. 1997, 1999).

4.4.1 Differential Scanning Calorimetry

Differential scanning calorimetry (DSC) is a thermoanalytical technique used to investigate the energetic states of materials. The heat flow to and from the sample, during a controlled temperature scan, is monitored and compared to an inert reference (Giron 2013). The DSC curves display the heat flow per gram of sample as a function of temperature and can be used to evaluate crystallization and polymorphic transitions from transition temperatures and enthalpy.

DSC can be conveniently used to confirm the solid nature of lipid particles by detection of a melting transition on heating. The DSC curves also provide information on melting enthalpy which can be used to quantify the amount of crystalline material present in the sample. This technique has been explored to characterize lipid nanoparticle formulations before processing (original dispersions) and after processing (freeze-dried powders and semi-solid formulations). DSC recrystallization studies indicate the possible problems that may arise when recrystallizing dispersed lipid materials. Such information can be useful to develop a thermal procedure that will ensure solidification of dispersed particles (Bunjes et al. 2001; Siekmann and Westesen 1996; Westesen and Siekmann 1997).

The melting temperature of nanosized lipid particles is often approximately 3–5 °C below the melting temperature of bulk lipid material. This phenomenon, commonly described as the Gibbs-Thompson effect, is due to the larger surface area-to-volume ratio of nanoparticles. The high surface energy associated with the nanoparticles results in an energetically suboptimal state which is responsible for lowering the melting point of the lipid material (Shah et al. 2014). Such a shift of melting point towards lower temperatures have been reported for lipid nanoparticles (Kovacevic et al. 2011).

Changes in physical properties of the formulation upon storage can be studied by DSC which can be used to monitor and quantify the polymorphic transitions and changes in crystallinity. The determination of absolute crystallinity of polymorphic lipids may be difficult and requires assignment of DSC transitions to different polymorphic forms. The colloidal state of particles may affect their thermal behavior which makes it difficult to compare the DSC data from lipid particles and bulk materials. SLNs in lower size-range have been reported to exhibit a decreased melting temperature. A decrease in melting temperature is often confused with the presence of a less stable polymorph. The melting transitions are usually broader in lipid nanoparticles than those in the bulk material (Bunjes et al. 2000; Trotta et al. 2003). For these reasons, DSC is best used to determine changes in physical characteristics with time, rather than absolute thermodynamic data of SLNs.

Determination of the recrystallization index (RI), or degree of crystallinity, can provide an estimate of the amount of recrystallized solid and is calculated from the DSC data using the equation (Eq. 4.3) (Kovacevic et al. 2011),

$$RI\ (\%) = \frac{\Delta H_{sample}}{\Delta H_{bulk\ lipid} \times C_{lipid\ phase}} \times 100 \tag{4.3}$$

where ΔH_{sample} is the melting enthalpy of the lipid nanoparticles, $\Delta H_{bulk\ lipid}$ is the melting enthalpy of the bulk lipid and $C_{lipid\ phase}$ is the concentration of lipid phase of the formulation. The RI is determined in cases where absolute crystallinity is not desired. Again, such measurements are used to monitor changes in crystallinity on storage. The degree of crystallinity of SLNs has been studied, for example, to investigate the influence on gelation tendency, occlusive properties and enzymatic degradation (Freitas and Müller 1999; Olbrich et al. 2002; Wissing and Müller 2002).

DSC can also be employed to investigate the interaction of drugs with the lipid material. A shift in melting transitions can be due caused by the eutectic behavior with incorporated drugs or liquid lipids. These interactions can also be reflected by changes in melting enthalpy. The presence of crystalline drugs in the lipid nanoparticle formulations can be confirmed by the occurrence of melting peaks of bulk drug in the DSC curve. However, not many researchers have reported the presence of crystalline drug (Cavalli et al. 1998; Frederiksen et al. 2003). The absence of peaks corresponding to the melting transition of drugs probably reflects incorporation of the drug into an amorphous or molecularly dispersed state (Anantachaisilp et al. 2010; Chen et al. 2006; Shah et al. 2014; Vivek et al. 2007). DSC has also been used to confirm the presence and solid state of lipid nanoparticles incorporated into semisolid formulations (de Vringer and de Ronde 1995; Wissing and Müller 2001, 2003).

4.4.2 X-ray Diffraction

X-ray diffraction (XRD) is based on measurement of diffraction patterns of X-rays on interaction with the crystal lattices in randomly oriented particles. The resultant diffractogram displays the intensity of reflected X-rays with respect to the angular position of the incident X-ray beams. The diffractogram provides a characteristic XRD pattern for a specific crystal structure under investigation that can be used in structure identification or differentiation of its polymorphic forms (Suryanarayanan and Rastogi 2002). XRD has been most commonly used in the characterization of SLNs to confirm the solid, crystalline nature of particles and to identify the polymorphic form of the lipid matrix after preparation (Cavalli et al. 1995; Jenning and Gohla 2000; Negi et al. 2013; Salunkhe et al. 2013; Teeranachaideekul et al. 2008).

The correct assignment of polymorphic forms in DSC curves is often a challenging task. A complimentary technique such as XRD provides additional information which may allow more unambiguous assignment of polymorphic forms. Processes such as phase separation may also be detected by XRD. The classic example is that of glycerides which occur in three polymorphic forms, each with a

different density. Each of these three forms has a distinct subcell packing of lipid chains. Specific to the polymorphic form, these chains tilt at a distinct angle with respect to the molecular glyceride layer. These polymorphic forms can be identified by XRD based on the wide angle X-ray reflections arising out of different subcell packing of lipid chains (Bunjes and Unruh 2007). Additionally, an intermediate polymorphic form β_i was also observed in complex triglyceride mixtures such as hard fats (Westesen et al. 1993, 1997).

The bulk saturated monoacid triglycerides are usually observed to be in the α and/or β form. Similar polymorphic forms are observed in triglyceride nanoparticles (Bunjes et al. 2003; Westesen et al. 1997). The occurrence of β′-modification is often observed when complex triglyceride colloidal systems are investigated. Such polymorphic forms also occur when triglyceride mixtures or partial diglycerides are employed in preparation of colloidal dispersions (Jenning et al. 2000b; Schubert and Müller-Goymann 2005; Westesen et al. 1997). XRD diffraction studies on glyceryl behenate nanoparticles also confirm the presence of the β′-form (Jenning et al. 2000b, c; Jores et al. 2003). SLNs based on solid paraffin and cetyl palmitate have also been shown to exhibit similar XRD patterns (Jenning and Gohla 2001).

Although DSC and XRD are often used to evaluate the state of the drug in colloidal dispersions, one should always remember that neither is well suited for detecting small amounts of foreign molecules in a lipid carrier. The detection limit of the drug depends on the relative amounts of the carrier matrix. Large signals from drug molecules may be superimposed by signals from the carrier matrix which makes it difficult to detect the presence of such moieties. The signals may be lost in the baseline when low concentrations of drug molecules are present. The Rietveld analysis, a peak fitting method, may then be used to find signals due to small amounts of crystalline drug, but such methods have seldom been used in lipid nanoparticle research (Bunjes and Unruh 2007).

4.4.3 Small Angle X-ray Scattering

Several studies have shown successful incorporation of drug molecules into SLN systems. It is less clear, however, as to whether the drug is really encapsulated or more simply co-exists as a drug nano-suspension. Small angle X-ray scattering (SAXS) is another important technique that can be used to supplement data from techniques such as PCS in the analysis of the phase-separated nano-materials. The SAXS patterns are useful to understanding of the internal arrangement of the lipid matrix, confirming the association of encapsulated drug—as shown in Fig. 4.10 (Castro et al. 2009; de Souza et al. 2012). SAXS can help understand the lipid-drug interaction in a drug-loaded SLN system. In combination with DSC, SAXS can assist in ascertaining the polymorphic forms (and/or crystallinity) of the SLN systems. Careful examination of SAXS patterns (presence of peaks) can provide details of any encapsulated drug in the crystalline or the amorphous region of the SLN system, and thus can be used to predict the stability of the system.

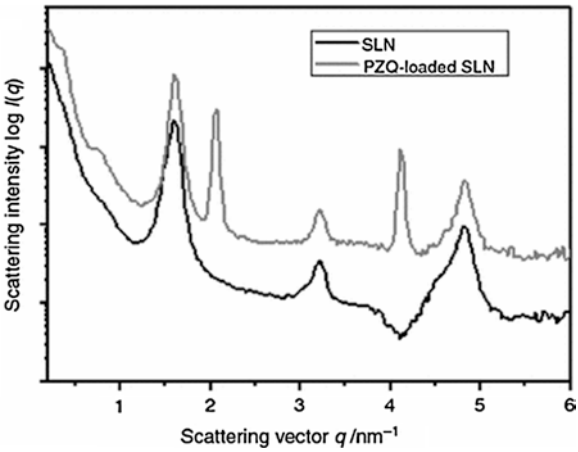

Fig. 4.10 The SAXS patterns observed for blank solid lipid nanoparticles and praziquantel-loaded solid lipid nanoparticles. Reprinted from J Therm Anal Calorim, de Souza et al. (2011), with permission from Springer

4.5 Co-existence of Addition Colloidal Structures and Interaction with Incorporated Drugs

The existence of other colloidal structures such as supercooled melts, micelles and liposomes in lipid nanoparticle preparations should also be considered. Lipid nanoparticles based on hard fat types show the presence of supercooled melts (Jenning et al. 2000a). Characterization and quantification of this aspect of lipid nanoparticles is often neglected. Moreover, the equilibrium of the complex colloidal system is often more fragile than the structure of the nanoparticles themselves, and therefore more commonly altered by formulation conditions such as the pre-treatment involved. Diluting the lipid nanoparticle dispersions with water often results in removal of surfactants, both from solution and thus altering the structure of the complex system, and also from the surface of the nanoparticles, resulting in crystallization and lipid modifications. There is thus a need for a technique which can simultaneously detect undesired colloidal species with comparable sensitivity but without much pre-treatment.

Although there is a vast amount of literature available on lipid nanoparticles, the knowledge related to the interaction of these colloidal systems with incorporated drugs is limited. Such interactions are typically drug-specific and may also be dependent on the composition and preparation technique of the lipid nanoparticles. It can be assumed that crystalline systems may be unfavourable for localization of drugs because the incorporated drug molecules will disturb the structure of the crystal lattice. Drug encapsulation depends on the lipid matrix employed in preparation of the nanoparticles and its crystalline and polymorphic status. Excess drug that is not encapsulated into the matrix may adsorb onto the particle surface or may precipitate as drug crystals in pure form or form drug suspensions or may get accommodated in other colloidal structures present in the dispersion. There is no method currently available that can determine the actual amount of drug incorporated within the nanoparticles or indicate the nature of the interaction between

the drug and the particles. Such information may provide a better understanding of its in vitro and in vivo performance. The use of spectroscopic techniques such as nuclear magnetic resonance (NMR) and electron spin resonance (ESR) provides a promising approach to evaluate the presence of other colloidal structures as well as the types of interactions between the drug and the carrier. ESR and NMR spectroscopy may also provide new insights into the characterization of lipid nanoparticle formulations.

4.5.1 Nuclear Magnetic Resonance

Nuclear magnetic resonance (NMR) can unambiguously identify the chemicals present in the sample and can yield information about the molecular structure, mobility, intermolecular distances and diffusion properties of lipid nanoparticles (Teeranachaideekul et al. 2008; Wissing et al. 2004).

NMR can be used to find associations between drugs and the lipid carrier systems. For example, immobilization of drugs such as diazepam, menadione and ubidecarenone by trimyristin-based lipid nanoparticles, was observed to be stronger in SLN dispersions than in corresponding emulsions (Westesen et al. 1997). It was, however, unclear whether the drug was accommodated into the nanoparticle or was adsorbed onto the surface.

Mixing different solid lipids often disturbs the lipid crystal structure, with little evident improvement in the loading capacities. Interaction of liquid lipids (or oils) with the solid lipid often improves drug loading. The mixing behavior of these lipids in the colloidal structures, their environment and arrangement, and their mobility may be studied from NMR experiments. NMR phenomena have been exploited to study molecular physics, crystalline and non-crystalline materials. Based on the principle of absorption and re-emission of electromagnetic waves by different NMR active nuclei in the magnetic field, NMR experiments provide valuable information that is often unattainable with other analytical techniques (Jenning et al. 2000a).

The key to structural analysis of nanoparticles by NMR is to attribute the NMR signals (arising due to different chemical shifts) to its characteristic molecules, thereby providing information on the environment and arrangement of a molecule or any of its observed components. The mobility of oil molecules, for example, is often reflected by the width of the NMR signals. Broad signals with weak amplitudes are indicative of restricted mobility. Molecules with sharp and intense signals have higher mobility. Figure 4.11 illustrates the proton NMR spectra of nanoemulsions and NLCs with increasing amounts of medium chain triglycerides.

A significant limitation to NMR is that it cannot detect solid molecules due to their very short relaxation times (Jores et al. 2003). Based on this principle, NMR was used to demonstrate the solid-like structure of *para-acyl-calix-arene* based SLNs (Shahgaldian et al. 2003). Although many researchers have focussed on the use of NMR for structural characterization of lipid nanoparticles, the full potential of this technique is yet to be explored.

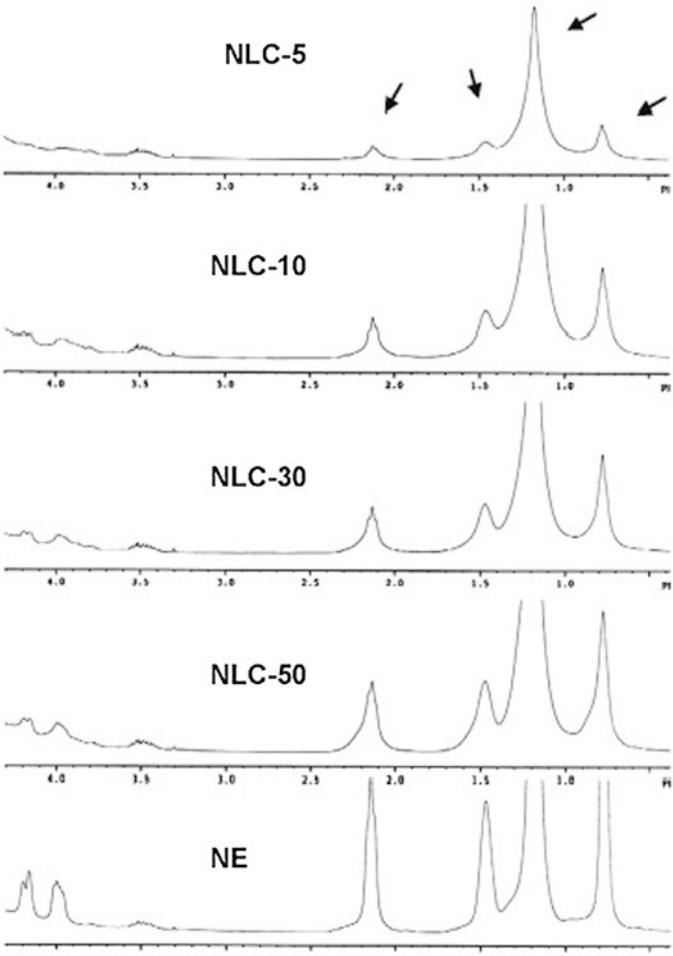

Fig. 4.11 Proton NMR Spectra of NLC prepared with increasing amount of medium chain triglycerides (5–50 %) compared to nanoemulsion. Reprinted from J Control Rel, Teeranachaideekul et al. (2008), with permission from Elsevier

4.5.2 Electron Spin Resonance

Electro spin resonance (ESR), alternatively referred to as electron paramagnetic resonance (EPR) is another technique that can be used to study the interaction of incorporated drug molecules with the lipid nanoparticles (Ahlin et al. 2000, 2003; Kristl et al. 2003). ESR is a powerful, versatile and sensitive spectroscopic technique. Unlike NMR, in which the signal arises by excitation of commonly occurring atomic nuclei, ESR involves excitation of unpaired, paramagnetic electrons. This technique involves the use of paramagnetic spin probes to investigate the

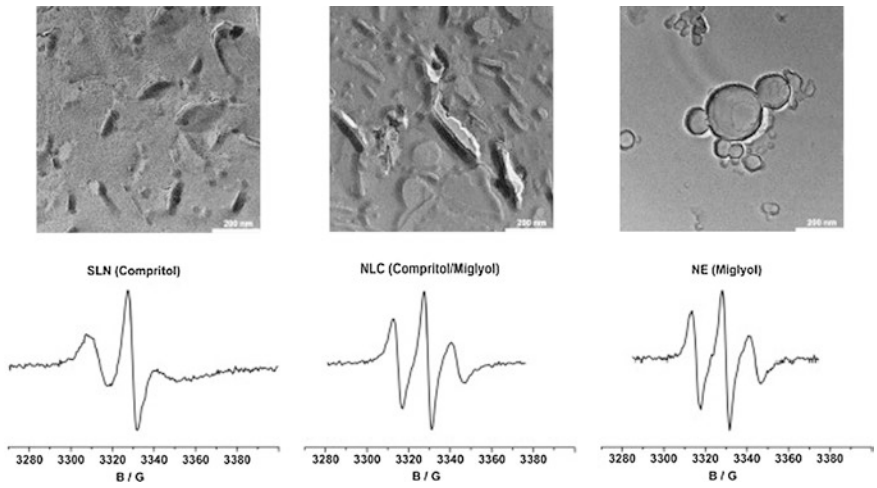

Fig. 4.12 Freeze fracture TEM images (*top*) and corresponding ESR spectra (*bottom*) of colloidal structures labelled with 0.025 % cholestane. (Composition: SLN (*left*)—glyceryl behenate, NLC (*middle*)—glyceryl behenate/Miglyol, Nanoemulsion—Miglyol). Reprinted from J Control Rel, Braem et al. (2007), with permission from Elsevier

signals arising from excitation of electrons in lipid nanoparticle dispersions. The technique involves probing the molecular structure and organisation of supramolecular systems and biomembranes. The ESR spectrum thus obtained provides information about the sample microviscosity and the micropolarity.

It has been previously shown that storage-induced crystallization of lipid in the lipid nanoparticles expels the probe out of the lipid phase into the outer aqueous phase (Jores et al. 2003). Accessibility of the lipophilic drugs to the aqueous phase is indicated by the rapid loss of intensity of ESR signals. The time-scale of the switch between the two phases can be exemplified by the ascorbic acid reduction assay (Jores et al. 2003). Reduction of the paramagnetic lipophilic drug to the ESR silent hydroxylamine by the hydrophilic ascorbic acid is the underlying principle behind this assay. ESR has also been used to study the interaction of SLNs with membranes (Kristl et al. 2003). Figure 4.12 shows TEM images obtained for various colloidal structures and supporting evidence provided by ESR.

4.6 Conclusions

A wide variety of tools have been employed to study complex lipid nanoparticle carrier systems. The corroboration of results obtained from different techniques may provide a better understanding of the structural and behavioral properties of these systems. However, in vivo investigation of particle characteristics is often difficult. The physicochemical properties of lipid nanoparticle dispersions are often dictated

by their chemical composition and the method employed in their preparation. The extrapolation of results of one colloidal system to another is thus difficult.

Lipid nanoparticle preparation is a well-established field of research and many researchers are attracted to this area of colloidal carriers. A number of questions such as the interaction of drugs with lipid carriers and the presence of other colloidal systems within the formulations are yet unanswered unambiguously. Both the introduction of new production procedures and variation of the lipid nanoparticle composition may affect system properties in unexpected ways. An intensive characterization scheme will be required to investigate the structure and behavior of complex colloidal carriers. This chapter has summarised the techniques available for such characterization.

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Chapter 5
Physicochemical Stability

Abstract Recent advances in lipid nanoparticle research suggest that colloidal carriers have great potential for administration of drug molecules. The use of physiological lipids in their matrices presents the advantages of biocompatibility and reduced toxicity. The lipids are known to influence drug encapsulation, particle morphology and drug release properties, as do other excipients such as surfactants, water and drug molecules. The rationale behind using lipid nanoparticles is the improved delivery of poorly water-soluble drugs. In addition, the lipid nanoparticles are expected to protect the drug from harsh biological conditions. The stability of lipid nanoparticles and the incorporated drug ensures improved drug efficacy. This chapter focuses on the physicochemical stability of lipid nanoparticle dispersions. The dispersion stability of pharmaceutical products is usually achieved by either of two stabilization mechanisms—electrostatic and/or steric stabilization. The destabilization mechanism, though undesirable when formulations are on shelf, can play a crucial role to in vivo applications where limited destabilisation is required for controlled drug release. A number of techniques such as water elimination or addition of specific stabilizers have been employed to optimize stabilization of lipid nanoparticle formulations. Although different measures have been taken to achieve the desired physicochemical stability, appropriate use of characterization tools to detect any destabilization in the system is necessary and will be discussed briefly here.

Keywords Physicochemical stability · Stabilization · Electrostatic · Steric · Destabilization · Optimization

5.1 General Considerations

The characteristic nanostructure of lipid nanoparticles provides a significant increase in the surface area to volume ratio (Yang et al. 2008). These effects result in distinctive in vitro and in vivo behavior of the lipid nanoparticles as compared to larger microparticles (Müller and Keck 2004; Rabinow 2004). Consequently,

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lipid nanoparticles have been extensively studied as potential drug carriers for enhancing the bioavailability of poorly water-soluble drugs by improving their water solubility or increasing the dissolution rate.

Protection of encapsulated drug molecules from the external biological environment is one of the major benefits of employing lipid nanoparticles. Chemical and biological degradation associated with the route of administration and from other constituents may negatively influence the carrier's stability. Stability is thus a critical facet that ensures drug safety and efficacy. Stability issues may also arise during storage (sedimentation, agglomeration, particle growth) or shipping. Such issues are, however, trivial in nanoparticle dry powders and hence, the focus is mostly on the stability of nanoparticle dispersions.

Lipids, surfactants, drugs and water are all major constituents of a lipid nanoparticle carrier system that can influence its stability. Although the use of physiological lipids reduces the toxicity associated with the carrier system, a minor chemical modification of these lipids may change the structure of the lipid nanoparticle. This may change the drug-loading capacity, surface or interface properties, or drug release properties. Sometimes, the drug itself may introduce destabilization into the systems. Table 5.1 summarizes the different influences that each of these constituents may have on the lipid nanoparticle system.

The unique features offered by lipid nanoparticles have prompted their extensive research for application in various dosage forms including oral (Das and Chaudhury 2011; Muchow et al. 2008), parenteral (Joshi and Müller 2009; Liu et al. 2011), ocular (Attama et al. 2008; Cavalli et al. 2002), pulmonary (Jaafar-Maalej et al. 2012; Liu et al. 2008), transdermal and cosmetic delivery systems (Liu et al. 2010; Souto et al. 2007). All of these dosage forms share common stability issues such as sedimentation, particle agglomeration or crystal growth, though their effects on the products may be quite different. For example, particle agglomeration is a major issue in parenteral drug delivery since it can cause blockage of blood capillaries and obstruct the flow of blood. Particle agglomeration in pulmonary drug delivery results in different deposition amounts per site and leads to a compromise in drug efficacy.

Table 5.1 Overview of the influences of different constituents used in the preparation of lipid nanoparticles

Influence of lipids
• *Drug encapsulation*—loading capacity, encapsulation efficiency, crystallinity and crystallinity index
• *Particle morphology*—particle size, particle shape, polydispersity index, zeta potential
• *Drug efficacy*—release
Influence of surfactants
• *Particle morphology*—particle size, particle shape, polydispersity index, zeta potential
Influence of drugs
• *Drug encapsulation*—loading capacity, encapsulation efficiency and crystallinity index
• *Particle morphology*—particle size, particle shape, polydispersity index, zeta potential
• *Particle efficacy*—release
Influence of water
• *Particle morphology*—polydispersity index

5.2 Stabilization Mechanisms

Stabilisation of the hot emulsion from which nanoparticles form and continued stabilisation of the nanoparticles once formed are likely to involve quite different mechanisms. The hot emulsions contains liquid particles which can deform and for which parameters such as zeta potential are dynamic rather than static, especially during particle interaction. Stability of the hot emulsion is likely to arise from the well-known Gibbs-Marangoni effect and is largely driven by the choice of surfactant (Walstra 1993). On cooling, however, the liquid emulsion solidifies to form a dispersion, and stability is now likely to relate to the well-known DLVO theory (and recent variants thereof) (Gambinossi et al. 2014; Ohki and Ohshima 1999).

The lipid nanoparticles distributed in the dispersion medium are in a constant state of random "Brownian" motion, and therefore frequently collide. If they "stick" on collision, then the overall surface area of the system will decrease, a process which lowers free energy, and consequently the system is thermodynamically unstable. Only if the particle size is extremely small, as is the case with microemulsions and micellar solutions, will the entropy caused by the sheer number of particles allow the system to be thermodynamically stable, Although nanosized particles may reach such an extreme small size, the particle size usually still remains large enough that the system is inherently thermodynamically unstable. The stability of the lipid nanoparticle dispersions thus depends on ensuring that particles do not "stick" on collision.

The forces that are operative during such collisions include:

- Van der Waals forces
- Electrostatic forces
- Solvation forces
- Electrical double layer compression
- Polymeric *inter-particle* bridging

A colloidal suspension can be stabilized in both aqueous and non-aqueous media through two common mechanisms, specifically electrostatic stabilization and steric stabilization. Electrostatic repulsion can be achieved, for example, by the addition of ionic stabilizers to the medium; steric stabilization can be accomplished, for example, by the addition of (large molecular) non-ionic stabilizers (Scheler 2012).

5.2.1 Electrostatic Stabilization

The electrostatic stabilization of colloidal particles can be explained by the classic Deraguin Landau Verwey Overbeek (DLVO) theory (Deraguin and Landau 1941; Verwey 1947). The DLVO theory assumes that colloidal stability is due to the additive effect of forces between particles. In particular, DLVO accounts for attractive van der Waals forces and repulsive electrostatic forces. The van der Waals forces of attraction between particles arise from electromagnetic attraction. The

energy of attraction (V_a) for two spherical particles, each with a particle diameter r and separated by a distance D is given by (Eq. 5.1),

$$V_a = -\frac{Ar}{12D} \tag{5.1}$$

where A is the Hamakar constant. The energy of attraction is proportional to the Hamakar constant and the particle size, and inversely proportional to the distance of separation.

The electrostatic repulsive forces originate from overlapping diffuse layers in the electrical double layer surrounding the two approaching particles in the medium. The electrical double layer (discussed in Chap. 4, Sect. 4.3) consists of two layers: (1) a Stern layer with counter ions attracted to the particle surface and (2) a Guoy layer which is a diffuse layer of ions, predominantly again counter-ions. The energy of repulsion (V_r) between two spherical particles, each with a particle diameter r is given by (Eq. 5.2),

$$V_r = \frac{32\pi r\varepsilon k^2 T^2 \gamma^2}{z^2 e^2} e^{-\kappa D} \tag{5.2}$$

where ε is the permittivity of the dispersed phase, k is the Botlzmann constant, T is the absolute temperature, γ is the reduced surface potential, z is the ionic charge, e is the charge on an electron, κ is the inverse Debye-Hückel length (reciprocal of the thickness of the electrical double layer) and D is the distance between two spherical particles. The energy of repulsion depends on the particle size, zeta potential, distance between the particles and dielectric constant of the dispersion medium. Ion concentration in the medium also has a significant influence on the energy of repulsion. The thickness of the electrical double layer decreases with increasing ionic strength of the medium, and consequently lowers the repulsive energy which can lead to aggregation (Nutan and Reddy 2009).

The total potential energy of particle-particle interaction (V_t) is the sum of the energies of attraction (V_a) and repulsion (V_r) generated from van der Waals attractive and electrostatic repulsive forces (Eq. 5.3).

$$V_t = V_a + V_r \tag{5.3}$$

Figure 5.1 illustrates a potential energy versus distance curve. The van der Waals attractive forces, as predicted from Eq. 5.1, are inversely proportional to the distance between two particles. By contrast, repulsive forces, as predicted from Eq. 5.2, decay exponentially with increasing inter-particular distance. It can thus be concluded that van der Waals attractive forces predominate at two specific distances (dependent on the system) and these result in a primary and secondary minima. Between these is a primary maximum which is dominated by electrostatic repulsive forces and is largely responsible for the observed stability in colloidal dispersions, i.e. such stability is brought about by a distance dependent barrier to close approach, somewhat akin to the activation energy which traditionally gives rise to kinetic (as opposed to thermodynamic) stability. The two particles are inseparable at a primary minimum where attractive forces are dominant. Such changes

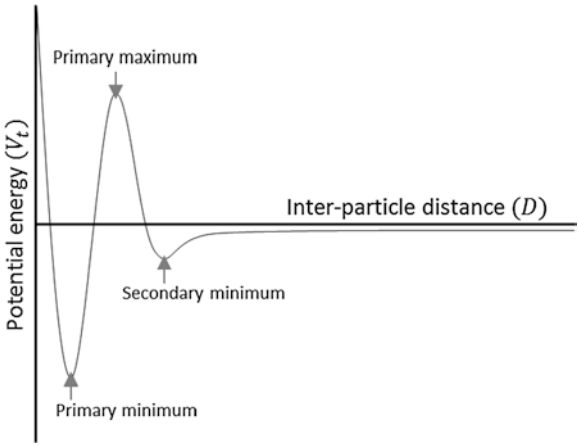

Fig. 5.1 Schematic form of potential energy versus distance *curve*, showing primary minimum (responsible for irreversible coagulation), primary maximum (responsible for kinetic stability of dispersions) and secondary minimum (responsible for reversible flocculation)

often cause irreversible changes such as particle crystallization in the system. The primary maximum is low and flat if the forces of repulsion are less than the van der Waals attractive forces. However, the particles remain in a dispersed state if their thermal energy is much higher than the primary maximum. The secondary minimum occurs at relatively large distances, and can cause loose particle flocculation, which can easily be reversed into dispersion by mechanical shaking. The particles repel each other and prevent coagulation if the thermal energy of the particles is larger than the secondary minimum. Not all features are apparent in all systems. The secondary minimum, for example, does not appear for small particles; it is operative only for larger particles (typically >1 μm and/or in high ionic strength).

Clearly, a number of significant advances to the original DLVO model have been made since its inception, for example the extended-DLVO (x-DLVO) model etc. (Van Oss et al. 1986). These include the introduction of solvation effects on the surface, as will be discussed in the next section on steric stabilisation. Whilst they are important to the quantitative determination of colloidal stability, they are complex and sometimes contradictory. The important aspects of the DLVO theory have not, however, changed and it allows an understanding of the parameters required to both predict and change colloidal stability. Specifically;

- Increasing the Hamaker constant (only possible by changing the lipid or substrate) will increase van der Waals forces thus decreasing stability.
- Increasing the surface potential (by, for example, adding a charged surfactant, altering the pH or decreasing the electrolyte concentration) will increase stability.
- Increasing the electrolyte concentration will decrease the zeta potential, thus decreasing stability, however it can, in some cases, also increase the surface potential giving the opposite effect.

Note that in all the above discussion, colloidal stability refers to the situation where the colloid remains dispersed and is therefore “stable” in the sense that it does not change. It does not refer to thermodynamic stability which would result in coagulation or flocculation and thus a change in the system.

5.2.2 Steric Stabilization

Steric stabilization is usually accomplished by the addition of large molecular sized non-ionic emulsifiers. The stabilizing mechanism can be attributed to the effect of solvation forces. Addition of water-soluble non-ionic emulsifiers to the nanoparticle dispersions can stabilize the nanoparticles due to their adsorption on the nanoparticle surface. They are adsorbed onto the nanoparticles through strong interaction of an anchor segment. The tail or stabilizing segment extends into the bulk medium and is strongly solvated. Thermodynamically steric stabilization can be indicated by estimation of Gibbs' Free Energy using the equation (Eq. 5.4),

$$\Delta G = \Delta H - T\Delta S \tag{5.4}$$

where ΔH is the enthalpy, T is the absolute temperature and ΔS is the entropy. Positive ΔG is indicative of a stable dispersion, while negative ΔG predicts particle aggregation.

Strong enthalpic interaction is responsible for good solvation between the solvent and the stabilizing segment of the emulsifier. The stabilizing segment cannot interpenetrate as the two particles approach towards each other due to its presence in a good solvent, and consequently force the bulk medium out of the inter-particle space. This keeps particles apart and results in steric stabilisation.

Steric stabilisation can also be induced by polymeric material, but large molecular non-ionic surfactants are more commonly used in SLNs.

Small molecular weight non-ionic surfactants can also help induce a stable system. In this case, the size of the stabilizer is not sufficient to result in full steric stabilisation, but may give partial steric stabilization to aid electrostatic stabilisation. More likely, the non-ionic surfactant can provide a solvation barrier to close contact, which modifies the DLVO model and has been well documented. The solvation barrier is best thought of as an additional force in the DLVO model such that modern DLVO variants include calculations of van der Waals forces, electrostatic forces and solvation forces.

5.3 Destabilization Mechanisms

5.3.1 Physical Stability

5.3.1.1 Dispersion Modifications

The lipid nanoparticle organization in a dispersion often changes with age, and such change is ultimately responsible for destabilising the dispersion. An understanding of the destabilising mechanisms is useful to consider prior to discussion of ways to measure that destabilisation and ultimate prediction of dispersion stability. A brief discussion of various destabilization mechanisms in dispersions is given here.

Phase Inversion
The phenomenon wherein the two phases of a colloidal system spontaneously invert is called phase inversion and can occur at a critical temperature, pressure or concentration. Whilst this does not necessarily result in emulsion destabilization, emulsions are thought to be at their weakest when this happens. A technique that involves heating the pre-emulsion and subsequently cooling it above the phase inversion temperature has recently been studied in the preparation of nanoemulsions (discussed in Chap. 3, Sect. 3.2.12). Note that this destabilisation mechanism will have relevance during formulation (in the hot emulsion) but will not have relevance once the particles have cooled to form an SLN.

Ostwald Ripening
The molecular diffusion of smaller particles into larger ones through the continuous phase is responsible for changes in particle size and size distribution. Such species flux can lead to particle growth or Ostwald ripening. Smaller particles dissolve more rapidly than larger particles (due to increased internal pressure), however their deposition rate back onto particles does not strongly favour the smaller droplets, so over time, larger particles grow and smaller particles disappear. Coalescence of small particles in the early stages of emulsion formation may also contribute to the disappearance of very small particles, although is a separate mechanism to Ostwald ripening. The end result is a decrease in polydispersity during the early stages of emulsion formation, and a loss of very small particles. Ostwald ripening is usually only relevant in the very early stages of emulsion formation and is again only relevant for the formulation (in the hot emulsion) of SLNs and will not be relevant to the formed SLN dispersion.

Coalescence
A collision between two droplets distorts the interfacial layer, and this distortion may eventually lead to rupture allowing the droplets to combine and merge into a single larger particle. Eventually this will lead to phase separation. Again, the phenomena is relevant to the hot emulsion formation stage and less relevant to solid, dispersed SLNs. The equivalent for solid dispersions is sintering, but this generally requires a high temperature leading to partial melting of the surface. Coalescence is an irreversible phenomenon.

Flocculation
The van der Waals suite of forces between particles leads to the formation of clusters or aggregates. Bridging between the particles is responsible for formation of larger structures. Although the particles are close to one another, they are separated by a finite distance, with water remaining between them. Each particle maintains its integrity, the energy "well" for flocculation is generally small, and thus it is a reversible phenomenon. The flocs that are formed can be easily redispersed often by simple shaking, if not with more vigorous mechanical agitation or ultrasonication. Flocculation relates to the secondary minimum as discussed in Fig. 5.1.

Coagulation
If particles are able to overcome their primary maximum (see Fig. 5.1) then they will be in close contact. Unlike flocculation, this process now involves tightly

bound particles with little to no water separating them, and is largely irreversible due to the deep energy well "stuck" particles are now in. Furthermore, because the particle surface is solid, they are not likely to coalesce so remain as discrete particles, but so tightly bound that they cannot be re-dispersed.

In both the cases of flocculation and coagulation, particles size now has two meanings—the intrinsic particle size of the individual particle and the effective particle size of the agglomerate (floc or coagulum). The effective particle size increasing results in creaming, sedimentation and/or gelling and results in dispersion instability.

Creaming and Sedimentation

Lipid nanoparticles either settle (sedimentation) or float (creaming) depending on their density relative to the density of the dispersion medium. Although flocculated dispersions do not necessarily result in serious instabilities, a settled or a creamed product is considered to be pharmaceutically and cosmetically inelegant, and may result in administration of an inadequate dose. Creaming or sedimentation, when the result of coagulation, is always serious.

The particles suspended in the dispersion are in a constant state of Brownian motion and collisions between the particles cause those particles to either settle or cream. The drag in the dispersion medium, however, causes the particles to resist settling. The resistance provided by the dispersion medium is proportional to the velocity of sedimentation. The gravitational force acting on the particle balances the resistance offered by the dispersion medium. The phenomenon of creaming or sedimentation can be explained according to Stokes' Law (Eq. 5.5),

$$V = \frac{d^2(\rho_1 - \rho_2)g}{18\eta} \tag{5.5}$$

where V is the velocity of sedimentation, d is the diameter of the particle, ρ_1, and ρ_2 are the densities of the particle and dispersion medium, respectively, η is the viscosity of the dispersion medium and g is the acceleration due to gravity.

According to Stokes' Law, the particle size, medium viscosity and the difference in densities of particles and medium are the main factors that influence the sedimentation rate of particles (Kim 2004). Decreasing the particle size or increasing the medium viscosity are the most common strategies to reduce particle sedimentation. The difference in densities of particles and dispersion medium also has an influence on the rate of sedimentation. Although the density of lipid nanoparticles cannot be changed, the density of the dispersion medium can be increased slightly by addition of density modifiers (such as mannitol and sorbitol). Since the density of the dispersion medium can rarely be increased above 1.3, it is practically impossible to eliminate the density difference (Nutan and Reddy 2009).

Dispersion instability can thus be mitigated by either (a) increasing the primary maximum (Fig. 5.1) leading to kinetic stability of the dispersion, or (b) slowing the rate of sedimentation or creaming.

5.3.1.2 Lipid Modifications

Previous reports suggest that lipid nanoparticle dispersions are more stable compared to other lipid colloidal carrier systems (Araújo et al. 2009; Müller et al. 2002; Saupe et al. 2006; Wong et al. 2012). Slow crystallization kinetics and polymorphism of the dispersed lipid can impart additional stability to dispersions and may well be the reason for this added stability. Thus crystallinity and polymorphism of SLNs, produced by hot homogenization and microemulsion techniques, is very important.

Crystallinity and Polymorphism of Lipids

The optimum performance of lipid nanoparticles is largely dependent on crystallization and polymorphism of lipid material. Crystalline solids have an orderly, repeated arrangement of atoms, molecules and/or ions. The additional stability exhibited by SLNs may be due to the absence of crystallinity and the presence of a polymorphic nature in the dispersed lipid material. Crystalline solids exhibit sharp melting points as they pass from solid to liquid state, with the melting point (as temperature rises) and the fusion temperature (as the temperature decreases) being similar, if not identical. The behaviour of transition points is thus of great interest when characterizing SLN formulations.

The formulation of SLNs from hot melt-dispersed lipid materials by hot homogenization and microemulsion methods is based on the use of polymorphic properties. SLNs are consequently formulated from lipid materials, for example glycerides, that are liquid at the formulation temperature, but solid at room temperature. The fusion temperatures of glycerides such as tristearin, tripalmitin, trimyristin and trilaurin are 73, 64, 56 and 47 °C, respectively. Note, as expected, that the fusion temperatures of glycerides with long fatty acid chains (tristearin and tripalmitin) are higher than that those with short chains (trimyristin and trilaurin). The fusion of the slightly less stable β-form of these triglycerides occurs at 68, 60, 53 and 43 °C, respectively. Recrystallization of these triglycerides in the α-form occurs at much lower temperatures; 51, 42, 28 and 11 °C, respectively (Westesen and Bunjes 1995). The fusion temperature actually encountered in SLN production can, therefore, vary according to the morphic phase produced, and may not be a sharp transition.

The colloidal size of triglycerides can also influence the solidification process, and therefore the fusion temperatures. Lipid nanoparticles melt at temperatures typically 3–5 °C lower than their bulk lipids (Bunjes et al. 2000; Westesen and Bunjes 1995). Under some circumstances, colloidal triglyceride dispersions (tristearin, tripalmitin, trimyristin and trilaurin) have been shown to recrystallize at 30, 21, 9 and −8 °C, respectively, approximately 20 °C lower than the bulk lipids (Westesen and Bunjes 1995). This study suggests that when lipid dispersions were cooled below their critical temperatures, particle recrystallization did not actually occur and emulsions of supercooled melts were formed. The supercooled melts are thermodynamically unstable and will gradually crystallize, leading to problems such as gelling and drug expulsion (discussed later under Gelation phenomenon). The majority of SLNs, however, are solid when first formed (Müller et al. 1996).

For genuinely solid SLNs, polymorphism remains an aspect that can cause physical degradation of solid dosage forms. Polymorphism is the ability of a solid material to exist in different unit structures in crystals, arising from a combination of different molecular conformations and packing. Despite the fact that the polymorphs have a similar chemical nature, their physical properties such as the melting points and the enthalpies are distinguishable (Bunjes and Unruh 2007), as exemplified above, and discussed below, for the α, β′ and β forms of glycerides.

Glycerides commonly used in the production of lipid nanoparticles exist in three polymorphic forms, in increasing order of stability: α, β′ and β. The most unstable α-form has a tendency to transform into a metastable β′-form that has better chain packing. The β-form is the most stable form of glyceride available. Conformational transition from the α to the β′ and consequent transition to the β-form is the preferred path for optimal packing of glycerides (Lawler and Dimick 2002). The unstable polymorph gradually transforms into the more stable polymorph during storage at elevated temperatures. During the process, the particles lose the initial spherical shape and form crystalline aggregates. For this reason alone, drug laden SLNs should be stored at low temperatures.

Lipid polymorphism influences the drug content and can result in drug expulsion on storage. Lipid molecules have a greater mobility in thermodynamically unstable morphic forms, making them more capable of incorporating drug molecules. This can be used to advantage with the inclusion of oils in the formulation of NLCs increasing mobility in the lipid system and thus the drug loading capacity (Bunjes et al. 1996; Jenning et al. 2000c). The crystallization of glyceryl behenate lipid nanoparticles can be delayed by inclusion of liquid medium chain triglycerides, consequently increasing the long term stability. The release of drug, which normally depends on the polymorphic transformation, was also delayed showing both improved emulsion stability and improved drug retention. Another example is that of the drug, Baicalein, which was able to be incorporated into an SLC through the addition of Vitamin E. The inclusion of Vitamin E, a liquid, in the formulation increases the number of imperfections and consequently reduces the partitioning of drug between the aqueous and lipid phases. Baicalein, which is prone to oxidation in aqueous environments was not expelled on storage, thus also protecting it from chemical integrity (Tsai et al. 2012).

Crystals with ordered lattices have high melting enthalpies and more energy is required to disturb that ordered arrangement. Lipid nanoparticles with increased melting enthalpies have been reported to exhibit a higher degree of drug expulsion (Tan and Billa 2014). The crystals rearrange themselves towards a higher thermodynamically stable configuration during storage, and this often leads to drug expulsion (Jenning et al. 2000b). As discussion, the polymorphic transition from β′ to β form reduces the amorphous regions in the crystal lattice which causes drug expulsion (Jenning et al. 2000a).

The mechanism of drug release has been linked to crystal structure by a number of researchers (Lukowski et al. 2000). An increased release of drug has been observed with polymorphic transitions in SLNs. The metastable polymorphic form is responsible for sustained release of drug. Changes in crystallinity

preferably towards a more perfect crystalline structure (evidence by increase in melting enthalpy) can thus be theorized to expel encapsulated drug on storage. Crystal reorientation has also been linked to changes other physical properties such as zeta potential (Freitas and Müller 1999).

Gelation Phenomena

Lipid nanoparticle dispersions have the potential risk of transforming into a viscous gel. The process, called gelation, is a very rapid and an unpredictable occurrence. Particle aggregation may occur as a result of loss of colloidal particle stability and precedes the gelling step. Typically, gelation results from long range particle interaction caused, for example, by polymeric material or large molecular weight non-ionic surfactants. These link particles in such a way that a three dimensional network across the entire sample is formed, and is more dependent on the nature of the cross-linking material (gelling agent) than the particle itself. Gelation is thus a function of the formulation more than a function of the nature of the particles within the SLNs. Gelation can also occur as a result of an ordered dispersion where particles are in sufficiently close contact that their random Brownian motion is restricted. In these cases, zeta potential is very important. Particles are repelled from each other as a result of the zeta potential, however if the dispersion is concentrated they cannot be repelled because other particles are present in every direction, and these are just as repelling. The result is a viscous ordered dispersion, akin to a gel. Physical characteristics, such as temperature and salt concentration are important.

Different factors stimulate the gelation phenomena such as (Awad et al. 2008; Freitas and Müller 1998; Helgason et al. 2008, 2009b):

- intense contact of the aqueous dispersion with surfaces (such as needle syringes)
- mechanical stress during sample manipulation and transport
- exposure to elevated temperatures or light or oxidising environments
- surfactants
- polymorphic transitions during storage conditions
- Ionic nature of drug

Most of these factors increase the kinetic energy of the particles, which increases the number of collisions between the particles making the system highly unstable. Gelling of nanodispersions is influenced by crystallinity and polymorphic transition. Surfaces that are in contact with the aqueous dispersions tend to stimulate crystallization or lipid modification in the nanoparticle suspension. Crystallization usually favours transition to a more stable form (i.e. β-form), thereby increasing the surface area of the particle (formation of platelets in the β-form). Surfactants fail to stabilize the system further and this leads to particle aggregation and/or gelation (Awad et al. 2008).

The stability of aqueous suspensions of SLNs has been investigated as a function of storage temperature, light and packing material. Introduction of any form of energy led to particle growth and consequent gelation. Particle growth was not observed in SLN dispersions stored under cold conditions in the dark. Storage of

the aqueous dispersion in an oxidising environment (such as regular air) caused instability of the formulation. This was prevented by storage in a nitrogen environment (Freitas and Müller 1998).

Many drugs have an ionic structure. Addition of ionic species can reduce the zeta potential, which is undesirable as the SLN dispersions are stabilized by electrostatic repulsion. High ionic strength can result in particle aggregation and thereby promote gelation (Choi et al. 2014). Prevention of gelling of lipid dispersions can be achieved by the addition of co-surfactants or storage of aqueous dispersions at lower temperatures, preferably in the dark or in a nitrogen environment.

Finally, although gelation is an indication of particle instability and therefore undesirable, gelation can also cause the system to become highly viscous, which in turn reduces the number of collisions within any given time and can paradoxically therefore increase stability by slowing down the processes of instability.

5.3.2 Chemical Stability

As discussed earlier, lipid nanoparticles protect drug molecules from degradation in the biological milieu. Chemical stability of lipid, phospholipid and encapsulated drug is an important aspect which influences the overall stability of lipid nanoparticles.

5.3.2.1 Drug Stability

The drug chemistry and its reaction mechanism are the major determinants of drug stability (Wu et al. 2011). Drugs with ester or amide linkages may undergo hydrolytic degradation. Drugs with amino groups are susceptible to oxidative degradation (Garad et al. 2010). Many research groups have looked into the chemical stability of drugs encapsulated into lipid nanoparticles. Lipid crystallinity is one of the critical parameters that dictate drug solubility, stability, dissolution and efficacy (discussed in “Crystallinity and Polymorphism of lipids”, Sect. 5.3.1).

5.3.2.2 Phospholipid Stability

Phospholipids are widely used as stabilizers in colloidal carrier systems. Phospholipids are susceptible to hydrolysis and lipid peroxidation which limit their use in pharmaceutical formulations. Hydrolysis of phospholipid often produces lyophosphatidylcholine and fatty acids. Lyophosphatidylcholine is a toxic chemical that has haemolytic properties. The chemical stability of phospholipids in emulsions and liposomes have been studied, however, their stability in lipid nanoparticle systems is not well established (Mengersen and Bunjes 2012). Lipid peroxidation is another major pathway responsible for phospholipid degradation.

Peroxidation of the unsaturated acyl chains in the phospholipid increases the permeability of the lipid bilayer. Lipid peroxidation of phospholipids produces chemical products with undesirable chemical characteristics.

5.3.2.3 Lipid Stability

Assuming that triglycerides are largely present in the core of the lipid nanoparticle, they are more protected from the environment and therefore less susceptible to degradation than the phospholipids that stabilize the nanoparticles. As such, it is generally assumed that triglycerides do not create any problems in the stability of lipid nanoparticles. Hydrolysis, however, is a major pathway in the degradation of triglycerides and may still at the surface, if not within the core, of the nanoparticle. The chemical stability of lipids used in production of SLNs is often neglected by many researchers.

Radomska-Soukharev (2007) have investigated the chemical stability of various lipids and surfactants used in the production of SLNs. A variety of SLNs were prepared and stored for a period of 2 years. Relative percentages of mono-, di- and triglycerides varied in different formulations. SLNs prepared with triglycerides were more stable than those prepared with mono- and diglycerides. The chemical stability of SLNs with an initial triglyceride content of 97 % was still slightly higher than 96 % after 2 years. However, in the case of SLNs with an initial mono- and di-glyceride content of 95 %, the lipid content was reduced to approximately 89–95 %.

5.4 Stability Measurements

Most of the destabilization phenomenon (flocculation, coagulation, and gelation) can be determined by visual observation. These phenomena lead to changes in viscosity of the final product, and even phase separation; however these are often present only after long storage times. There is great interest in methods which predict ultimate long term storage stability without the need to wait for long times. Two clear strategies are generally used—(1) artificially increase destabilisation by processes such as increased temperature, and (2) the use of characterization tools (described in Chap. 4) which can detect such destabilization mechanisms before instabilities are clearly observable.

Stability testing at various temperatures is very common for pharmaceutical products, particularly emulsions, but also for dispersions such as SLNs. In general, formulations are allowed to age at a low temperature (typically 5 °C), room temperature (usually 25 °C) and elevated temperatures (e.g. 40 °C). The philosophy is that increased temperature increasing the collision rate, thus mimicking longer shelf time. A formulation which may last for, say 2 years, at room temperature is predicted to only last, say 3 months, at 40 °C.

Both storage at high temperatures, and at low temperatures are required, however it is often not understood why the lower temperature is required. Figure 5.1

can be used to illustrate the explanation. Particles trapped within the secondary minimum will tend to flocculate and this will cause dispersion instability. If, however, the temperature is increased, then the particles will have greater kinetic energy and can now "escape" the secondary minimum and become stable. Increasing the temperature has thus cause an artificial increase in stability. Testing at lower temperature will ensure this is not the case and prevent falsely believing a good dispersion has been formulated. Figure 5.1 also illustrates a second flaw in traditional temperature testing. Dispersions which are just below the primary maximum at room temperature will become unstable at higher temperatures because particles have more kinetic energy at the higher temperature and can thus overcome the primary maximum, leading to coagulation in the primary minimum. Thus a dispersion, which could remain stable indefinitely at room temperature, is unstable at higher temperatures, not because there are a greater number of particle collisions, but because particles have greater kinetic energy (Freitas and Müller 1998; Shah et al. 2014; Vivek et al. 2007).

5.4.1 Physical Stability

5.4.1.1 Modification of Size

Particle size is often used to characterize lipid nanoparticles. The importance of particle size has been discussed earlier (Chap. 4, Sect. 4.1). A number of factors have an influence on the mean diameter of lipid nanoparticles such as the excipient composition, drug, production procedure, process variables, sterilization, dispersion medium, storage conditions and many more. Each of these factors has been discussed earlier and is related to crystallization processes and coalescence (of the hot emulsion during formulation). For the dispersion, particle size will generally increase if any destabilisation mechanism is present. An increased particle size is generally observed before the appearance of any macroscopic changes. Particle size, thus, is an important indicator of physical stability. Some researchers have used increase in particle size to predict poor SLN stability (Freitas and Müller 1998; Kovacevic et al. 2011, 2014; Vivek et al. 2007).

5.4.1.2 Modification of Zeta Potential

The zeta potential is one of the fundamental parameters that play an important role in colloidal stability against particle aggregation (Heurtault et al. 2003), and was discussed in Chap. 4, Sect. 4.3. The measurement of zeta potential thus provides a good insight into the likelihood of dispersion, aggregation or flocculation.

The zeta potential can be used as an indicative tool to predict the shelf life of the colloidal dispersions. As a general rule, zeta potentials greater than about |30| mV give electrostatically stabilized systems, with the optimum zeta potential for electrostatic stabilization being greater than |60| mV. Limited flocculation (short-term

stability) can be observed between |5| and |30| mV. Zeta potentials below |5| mV often result in particle coagulation (Heurtault et al. 2003; Wu et al. 2011). A minimum zeta potential of approximately |20| mV is desirable for dispersions stabilized by a combined steric and electrostatic effect (Mitri et al. 2011; Tamjidi et al. 2013). There is no convenient value as zeta potential for systems stabilized with steric stabilizers only to characterize its extent (Bunjes and Siekmann 2005).

Similar to the measurement of particle size, changes in zeta potential on storage generally indicate pre-destabilisation mechanisms and can predict poor long-term dispersion stability even in systems which are initially stable. This is particularly the case where drugs are incorporated into the system—long term destabilisation of the drug may result in destabilisation of the dispersion and can be reflected in changes to parameters such as zeta potential. So a change in value can be just as important as the absolute value itself.

5.4.1.3 Modification of Crystallinity and Polymorphism

The effect of lipid modifications on drug incorporation has been well studied. Well established techniques such as DSC and XRD have been used for characterization of lipid modifications and show that variation in the behavior of lipid nanoparticle dispersions is due to structural differences within them. Marked supercooling is often observed in lipid nanoparticle systems. It is now clear that supercooled melts are emulsions and not lipid nanoparticle dispersions. However, the major difference between the two is the ability to crystallize. Crystallization may lead to several physical instabilities; thus, careful evaluation of this is necessary. The critical number of crystallization nuclei required to initiate crystallization is unlikely to develop in small droplets of emulsions. Therefore, supercooled melts tend to form more readily with decreasing particle size. Characterization by DSC and XRD of triglyceride dispersions (particularly with lower melting points such as trilaurin or trimyristin) stored at room temperatures did not show any melting point transitions (due to heating) in the DSC curve or deviation in the X-ray reflections. Proton-NMR also revealed the presence of liquid triglycerides in the lipid matrix. NMR allows a more rapid and non-destructive structural analysis of the colloidal systems, showing the presence of supercooled melts. The presence of systems having a high tendency to form supercooled melts was evident from these studies (Bunjes and Unruh 2007; Bunjes et al. 1996; Westesen et al. 1997).

5.4.1.4 Optical Analysis by Turbiscan® Lab

Apart from particle sizing, physical destabilization in a system can be detected by the naked eye, optical microscopy and turbidity measurements. These techniques, however, require sample dilution which may reduce the accuracy of absolute values. The Turbiscan® is a trade-mark device where users by-pass the need for absolute values and look, instead, for changes in particle size (reflected by a change in turbidity) (Araújo et al. 2011).

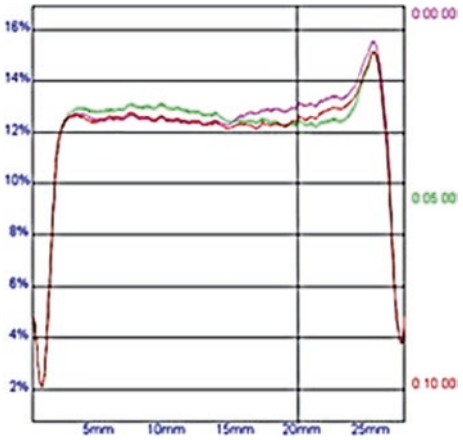

Fig. 5.2 Backscattering profiles of solid lipid nanoparticles prepared by ultrasonication technique. Reprinted from Colloid Surface B Silva et al. (2011), with permission from Elsevier

The principle of Turbiscan® is based on analysis of multiple scattering of light by concentrated dispersions. Turbiscan® is equipped with a near-infrared monochromatic light source ($\lambda = 880$ nm) and two synchronous backscattering (BS) and transmission (T) detectors. Turbiscan® measures the BS and T signals arising from backscattering and crossing of samples, respectively. These signals denote the fluctuations on particle volume and size which represent particle migration (creaming, sedimentation) and particle aggregation (coalescence, flocculation), respectively. Particle migration is reversible wherein the particles can be easily redispersed by mechanical stirring, and particle aggregation is often irreversible (Silva et al. 2011). Figure 5.2 shows an illustrative backscattering profile of SLNs. It shows BS signals arising from backscattering of samples. Deviation within ± 2 % is often taken as an indicator of stable formulation.

5.4.2 Chemical Stability

The biological performance of lipid nanoparticle dispersions is highly dependent on the chemical stability of the incorporated drug molecules. Measurement of chemical stability is essential to calculate the encapsulation efficiency and drug loading of the lipid nanoparticle system. The degradation products of drugs may be potentially toxic, and therefore, it is necessary to investigate the chemical stability of drugs.

Chemical stability of lipid nanoparticles can usually be measured by two different approaches—destructive and non-destructive. Destructive methods rely upon dissolution of carrier system in organic solvents to release the drug. Quantification of the drug is usually performed by spectroscopic or chromatographic methods (Jia et al. 2012; Liu et al. 2011; Nik et al. 2012; Tsai et al. 2012).

Although these assays are simple, the quantified drug is inclusive of both encapsulated and unencapsulated drug molecules. Organic solvents such as methanol, acetone, acetonitrile or tetrahydrofuran that may be used for destruction of

carriers may themselves influence the chemical stability of the drug. Spectroscopic methods are ineffective at detecting the presence of interfering entities in the sample while chromatographic methods are efficient in determining the presence of degradation products as well as any impurities from the carriers.

Non-destructive assays rely on changes in physical characteristics such as color or absorbance. These methods are quite simple and avoid destruction of the drug carriers to release the drug. However, such measurements may be difficult in the presence of any crystallized drug molecules present in the dispersions (Helgason et al. 2009a; Mitri et al. 2011).

5.5 Optimization of Stability

Armed with a knowledge of the destabilisation mechanisms, and the methods which can be used to measure them, we can now focus on methods for optimising dispersion stability, both in terms of shelf-life and in terms of their passage through biological systems (assuming the requirement is the targeting of specific body sites).

5.5.1 Physical Stability

5.5.1.1 Steric Stabilization

Steric stabilization in lipid nanoparticles can be obtained by coating the nanoparticles with steric stabilizers such as Pluronics, also known as stealth agents. The lipid nanoparticles coated with stealth agents exhibit high stability against electrolytes (Heurtault et al. 2003). Physicochemical characteristics of colloidal carriers such as size, charge and hydrophobicity aid in their easy recognition by macrophages. Surface hydrophobicity of carriers can be improved by coating them with stealth agents such as Poloxamer and PEG agents. This prevents their recognition by the reticuloendothelial system and increases the circulation time of nanoparticles (Jia et al. 2012). Poloxamer 188 has been shown to prevent particle aggregation of NLCs and reduce instability by steric stabilization (Han et al. 2008).

5.5.1.2 Electrostatic Stabilization

Electrostatic stabilization in lipid nanoparticles is often achieved by addition of ionic surfactants to the formulation ingredients. The addition of ionic surfactant such as sodium deoxycholate to the aqueous phase of the formulation often gives negative charge to the polar group of the surfactant (Kumar and Randhawa 2013). An ionic layer surrounding the particle surface increases its zeta potential, thus preventing close approach of particles and maintaining good dispersion stability. It should, however, be acknowledged that any changes to the charge of the particle may influence its biological application.

5.5.1.3 Incorporation into Creams or Hydrogels

Earlier discussion on lipid crystallization and polymorphism emphasised the fact that crystal transformation is a major cause of drug expulsion on storage. Incorporation of lipid nanoparticles into semi-solid systems such as hydrogels or creams have been looked upon as a promising strategy to slow down the rate of crystal transformation and achieve long term stability (Souto et al. 2004). It will also decrease the rate of particle collision, increasing dispersion shelf life. The release of drug from lipid nanoparticles is expected to be slow due to the solid core of the nanoparticle. The release of drug can be further delayed by incorporating the lipid nanoparticles into hydrogels (Silva et al. 2012). The increase in particle size of lipid nanoparticles in hydrogels is often related to adsorption of gelling agent. It, however, does not impair its physical stability (Hao et al. 2014).

5.5.2 Chemical Stability

5.5.2.1 Antioxidants

Oxidation is the major pathway for degradation of phospholipids, lipids and drug molecules. The exclusion of oxygen from storage vials may prevent oxidation of formulation components. This can be done by replacing oxygen in the headspace with nitrogen gas (Teeranachaideekul et al. 2007). Addition of antioxidants such as α-tocopherol, butyl hydroxyl toluene (BHT) and butyl hydroxyl anisole (BHA) can also be used to enhance the chemical stability of formulations against oxidation. Inclusion of anti-oxidants during the production improved chemical stability of all trans retinol-loaded lipid nanoparticles (Jee et al. 2006). The use of a combination of antioxidants often imparts improved stability over the use of single antioxidants (Teeranachaideekul et al. 2007).

5.5.2.2 Water Elimination

Chemical instability due to hydrolysis can be prevented by elimination of water in the sample by spray-drying or freeze-drying. Freeze-drying is the most common technique applied for elimination of water from samples. However, the process can generate stress and destabilize the colloidal systems (Abdelwahed et al. 2006). Several sugars have the ability to conserve the properties of lipid carriers and overcome stability issues (Ohshima et al. 2009; Soares et al. 2013; Varshosaz et al. 2012). Freeze-drying often influences the particle size of the product (once re-dispersed); however optimization of freeze-drying processes can minimize such effects. Factors such as the type of cryoprotectants, lyophilization process, pH of dispersion, interaction of cryoprotectants and nanoparticles and surfactants may decide the success of the freeze-drying process (Abdelwahed et al. 2006). Trehalose has been found to be the most effective cryoprotectant in preventing particle

aggregation in freeze-dried lipid nanoparticles (Zimmermann et al. 2000). Other sugars such as mannitol, sucrose, fructose, glucose and sorbitol have also been used.

An alternative method for elimination of water involves converting a liquid dispersion into a dry powder by a process called "spray drying". The shear forces involved in the process may destabilize the system. Exposure of the sample to the elevated temperatures for drying is very short; the thermal stress on the material is dramatically reduced due to the latent heat of vaporization. There is always a risk, however, of melting of lipids which can alter the particle size of the nanoparticles (Blasi et al. 2013).

5.6 Conclusions

Physicochemical stability is an important consideration in the development of lipid nanoparticle formulations. The stability assessment of lipid nanoparticle formulations involves multi-technique investigations. The first major step in the quest to achieve desired physicochemical stability is the appropriate selection of formulation ingredients. The composition of formulations has an implied influence on various parameters, including particle size, zeta potential, drug encapsulation efficiency, loading capacity, crystallinity and drug release.

Finally, it should be acknowledged that the desired formulation stability is always a compromise between storage stability (shelf-life) and the destabilization mechanism that modulates the release of drug in biological systems. The goal, then, is not necessarily to create the most stable dispersion.

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