2 PRINCIPLES OF DERMATOLOGICAL FORMULATIONS

As reported in the previous chapter, in the present thesis it has been essentially concerned the technological and biopharmaceutical properties of topical formulations based on SLN and NLC. In addition, the basic science of how to formulate effective topical and dermatological drugs so that they can be released from their vehicle and penetrate the skin to reach their site of action has also been focused. Therefore, the pharmaceutical mode of action of the selected model drugs, or the detailed biochemistry, anatomy, and pathology of the disease state, has not been directly described. However, in order to design safe and effective formulations to treat skin injuries, a rational approach to topical and dermatological formulations will be given in the present chapter.

2.1 Anatomy and physiology of the skin

The human skin comprises two distinct but mutually dependent tissues, the stratified, avascular, cellular epidermis and an underlying dermis of connective tissue [218]. At the bottom of the dermis lies the fatty, subcutaneous layer which is called hypodermis. A simplified schematic cross-section of the skin is shown in Fig. 2.1.

The epidermis varies in thickness, depending on cell size and number of cell layers, ranging from about 0.8 mm on the palms and soles down to 0.06 mm on the eyelids. Cells which provide epithelial tissue differ from those of all other organs, once they ascend from the proliferative layer of basal cells, changing in an ordered fashion from metabolically active and dividing cells to dense, dead, keratinized protein.

In the thick epidermis of the palms of the hand and soles of the feet, there are five typical layers (strata). Starting with the outermost layer, they comprise the stratum corneum, stratum lucidum, stratum granulosum, stratum spinosum and stratum basale. The strata spinosum and basale together are known as the stratum germinativum once they generate new cells. In parts of the body other than the palms and soles, only the stratum corneum and stratum germinativum are regularly present. These layers will be briefly described starting from the innermost layer.

In the stratum germinativum, the basal layer is composed of basal cells, which are nucleated, columnar and about 6 μm wide, with their long axis at right angles to the dermoepidermal

junction, connected by cytoplasmic intercellular bridges. Mitoses of the basal cells constantly renew the epidermis and this proliferation in healthy skin balances the loss of dead horny cells from the skin surface, thus the thickness of the epidermis remains constant. Therefore, the total turnover time, from the basal layer to shedding, averages 28 days in healthy skin. The basal cell layer also includes melanocytes, which produce and distribute melanin granules to the keratinocytes in a complex interaction.

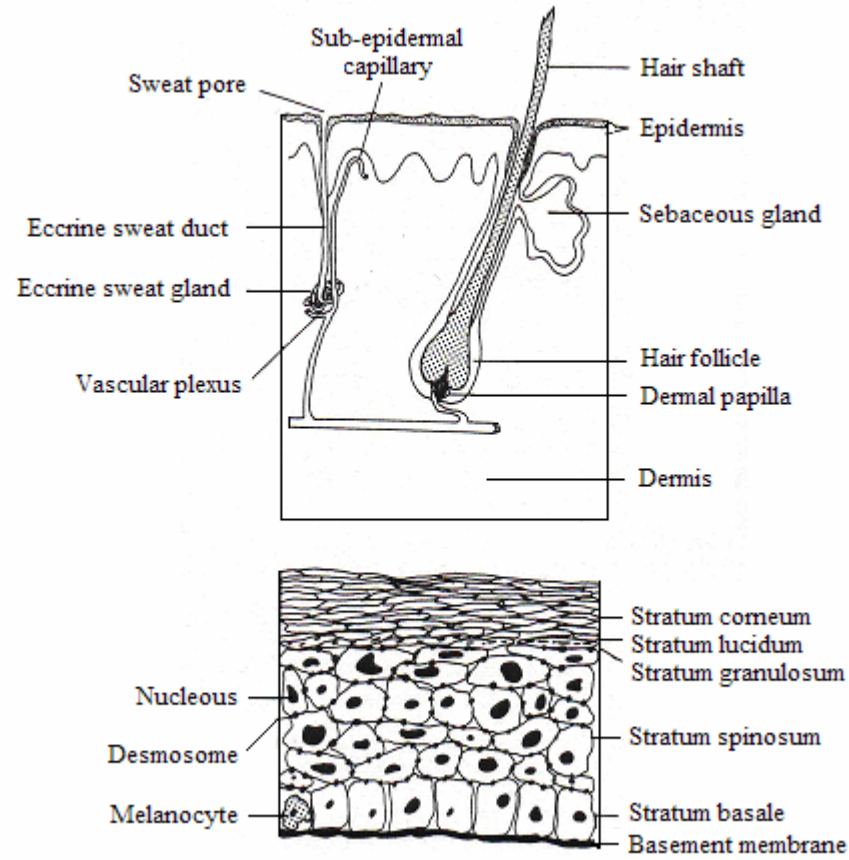


Fig. 2.1: Schematic cross-section of the skin. At the top of the figure the full thickness of the skin is described and below an expansion of the epidermis is illustrated (modified after Barry [218]).

Below the basal cell layer lies the complex dermatoepiedermal junction, which constitutes an anatomic function unit, where four components can be identified, i.e. the basal cell plasma membrane with its hesmidesmosomes, the lamina lucida, the basal lamina, and the fibrous components below the basal lamina, which include anchoring fibrils, derma microfibril bundles, and collagen fibrils. This basement membrane corresponds to the fibrous zone below the basal lamina. As the cells produced by the basal layer move outward, they change both morphologically and histochemically. The cells flatten and their nuclei shrink. They have a polygonal shape and are interconnected by fine prickle cells. Each prickle encloses an

extension of the cytoplasm, and the opposing tips of the prickles of adjacent cells adhere to form intercellular bridges – the desmosomes. These links maintain the integrity of the epidermis. Between the desmosomes a capillary space full of tissue fluid separates neighbouring cells and the void permits nutrients and oxygen to pass outward.

The stratum lucidum appears only in the palms of the hands and soles of the feet, an anatomically distinctly, poorly staining hyaline zone forms a thin, translucent layer immediately above the granular layer, acting as a protective shield against the ultraviolet (UV) rays of the sun and preventing sunburns.

At the final stage of differentiation, epidermal cells construct the most superficial layer of the epidermis, the stratum corneum, which is a flat, relatively thick layer of dead cells arranged in parallel rows. The cells lie tangential to the skin surface and interdigitate their lateral edges with adjacent cells so as to form cohesive laminae. These cells contain keratin, and are also the final repository of the end products of epidermal metabolism. They enclose sebaceous and sweat gland secretions in a highly organized structure. This layer plays a crucial role in controlling the percutaneous absorption of drug molecules.

The dermis, at 3 to 5 mm thick, is much wider that the overlying epidermis and it is considered the bulk of the skin [218]. The dermis consists essentially of a matrix of connective tissue woven from fibrous proteins (approximated composition: collagen 75%, elastin 4% and reticulin 0.4%), which embed in an amorphous found substance of mucopolysacharide providing about 20% of the mass. Blood vessels, nerves and lymphatics cross this matrix and skin appendages (eccrine sweat glands, apocrine glands, and pilosebaceous units) penetrate it. It can be divided in two parts, a superficial, thin, papillary layer composed of narrow fibres, which forms a negative image of the ridged lower surface of the epidermis, and a thick underlying reticular layer made of wide collagen fibres. The dermis also abounds with elastic fibres which stretch relatively easily and which revert to their original shape when the stress subsides. The elastic fibres form a framework in the dermis, so that the mechanical properties of connective tissues depend on the presence of both collagen and elastic fibres.

The dermis needs a rich blood supply which regulates temperature and pressure, delivers nutrients to the epidermis and removes waste products, mobilizes defence forces, and contributes to skin colour. Branches from the artery network (the arterial plexus) convey blood to the hair follicles, the sweat glands, the subcutaneous fat, and the dermis itself. The blood supply reaches to within 0.2 mm of the skin surface, so that it readily absorbs and

systematically dilutes most substances which penetrate past the stratum corneum and the viable epidermis. The vascular surface available for the exchange of materials between local tissues and the blood is about 1-2 cm^2 per cm^2 of skin surface with a blood flow rate of about 0.05 ml/min/cm^3 of skin. Of particular relevance to biopharmaceutical studies is the fact that this generous blood volume usually functions as a sink with respect to the diffusing molecules which reach it during the process of percutaneous absorption. This sink condition ensures that the penetrate concentration in the dermis remains near zero and therefore the concentration gradient across the epidermis is maximal. As the concentration gradient provides the driving force for diffusion, and abundant blood supply assists percutaneous absorption.

The hypodermis (subcutaneous fat) spreads all over the body as a fibrofatty layer, with the exception of the eyelids and of the male genital region [218]. The sheet of fat lies between the relatively flexible skin and the unyielding, deep fascia, and its thickness varies with the age, sex, endocrine, and nutritional status of the individual. The cells manufacture and store lipids in large quantities and bundles of collagen fibres weave between aggregates of fat cells providing flexible linkages between the underlying structures and the superficial skin layers. The hypodermis is also responsible for thermal barrier and mechanical cushion. It is a site of synthesis and a depot of readily available high energy chemicals.

2.2 Drug penetration through the skin

As exposed in the skin anatomy and physiology section, one can say that skin is particularly effective as a selective barrier for penetration (or elimination) of a diverse range of substances [219]. The epidermis is the major element in this control as illustrated by the evidence that most of small water-soluble non-electrolytes can diffuse into the capillary system as thousand times more rapidly when the epidermis is absent, damaged or diseased than when it is present and intact.

From the anatomical considerations exposed above, it is concluded that in general the principal barrier function of the epidermis resides almost entirely in the stratum corneum. For a material absorped percutaneously, the process is a sequence of deposition onto the stratum corneum, diffusion through the living epidermis and the passage through the upper part of the papillary dermis. The viable tissues may metabolize the drug, particularly the epidermis, which contains most of the catabolic enzymes that either render a topical drug inactive or activate a pro-drug. The important role, which living skin plays as an active metabolizing

tissue and, in particular, how this process affects the topical bioavailability of a drug, are subjects that are only now being treated fundamentally. The papillary layer of the dermis contains so many permeable capillaries that it is highly probable that most molecules enter the microcirculation soon after leaving the epidermis. Thus, the average total residence time of a drug species in the dermal aqueous phase may only be of the order of a minute. The deeper layers of the dermis should not, in general, influence percutaneous absorption.

A further problem arises if the drug is very lipophilic. After the molecule passes the horny layer, it suddenly meets an aqueous interface in which it is poorly soluble. The thermodynamic activity of the diffusing species immediately below the barrier may then be relatively high and approach that in the barrier itself. The activity gradient from the stratum corneum to the viable tissues falls and consequently so does the flux. The rate-determining step may become the clearance rate from the barrier rather than the penetration of the barrier.

The permeation process to the diffusion of a substance through a three-ply membrane, i.e. horny layer, viable epidermis, and dermis, with the microcirculation can be idealized as an infinite sink. If we apportion separate diffusional resistances to the skin layers, the total diffusional resistance of the composite skin barrier in steady state permeation can be described using the following equation [220]:

$$\sum R_i = \frac{h_{SC}}{D_{SC}\,K_{SC}} + \frac{h_E}{D_E\,K_E} + \frac{h_D}{D_D\,K_D} \qquad (1)$$

where R_i is the diffusional resistance of the i^{th} layer and the subscripts SC, E and D refer to stratum corneum, viable epidermis and papillary layer of the dermis, respectively.

The dermis and viable epidermis are extensively hydrated and diffusion coefficients are typical of liquid-state diffusion. In the dermis, molecules probably move within water-filled interstices. In contrast, the stratum corneum is a semi-fibrous structure which is characterized by a fibre or semi-solid diffusivity. The diffusional resistance of the stratum corneum to water is approximately 1000 times that of either the viable epidermis of the superficial region of the dermis. Polar solutes (other than water) and larger molecules usually have smaller diffusion coefficients than that of water, and the stratum corneum becomes even more dominant in the total permeation process. In general, the conclusion that the horny layer provides the rate-limiting barrier for diffusion is true for the entire class of water-soluble substances.

The viable layers of the skin are relatively more significant barriers to the penetration of non-polar molecules than of polar molecules, but they are still insignificant for *in vivo* percutaneous absorption (they represent only about 4% of the total diffusional resistance).

Thus, for polar and non-polar molecules alike, the stratum corneum provides the rate limiting barrier to percutaneous absorption.

The lipid organization of the stratum corneum is highly responsible for the transport properties of the skin. A layered structure of epidermal lipids has been proposed [220], and is schematically illustrated in Fig. 2.2.

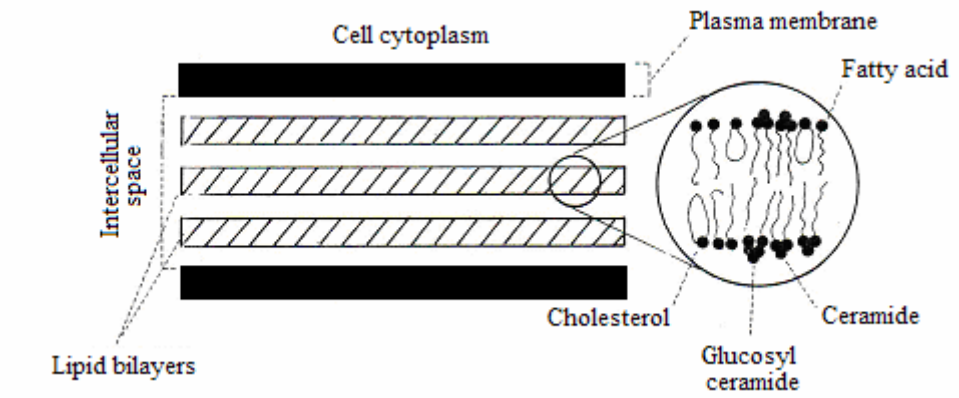


Fig. 2.2: Schematic representation of the layered structure of epidermal lipids (modified after Osborne [220]).

The polar head groups of the lipids are gathered in layers with the non-polar chains pointed in opposite directions forming layers of methyl groups in the plane where the hydrocarbon ends. Fig. 2.2 shows that not all the lipids are positioned with their polar groups localized in the polar layer. In fact, some of them are actually localized in the region between the methyl groups. In addition, those patterns also provide information with which is possible to estimate the penetration by water into the space between the lipid molecules. The diffusion in liquid crystals is highly anisotropic. The essential information for the problem of transport across the stratum corneum is that the diffusion parallel to the layers is fast, the same magnitude as in a liquid. Conversely, perpendicularly to the layers the diffusion is one or two magnitudes lower. A layered structure, such as that in the lipid part of the stratum corneum, is not a perfectly organized array of layers parallel to the skin surface but, instead, a series of dislocations always occur, as schematically shown in Fig. 2.3. Therefore, the diffusion coefficient is the gross one for a partially organized lamellar structure.

The analysis of the possible ways in which a topical drug may reach the viable epidermis and the upperdermis, and in what relative amounts, is quite complicated. When a drug molecule moves onto the intact skin delivered from a vehicle, it first contacts with the sebum, cellular debris, bacteria, and other exogenous materials which cover the skin. In general, a molecule may penetrate to the viable tissue below the horny layer via two potential routes of entry to the subepidermal tissue, i.e. transepidermal route and transappendageal route.

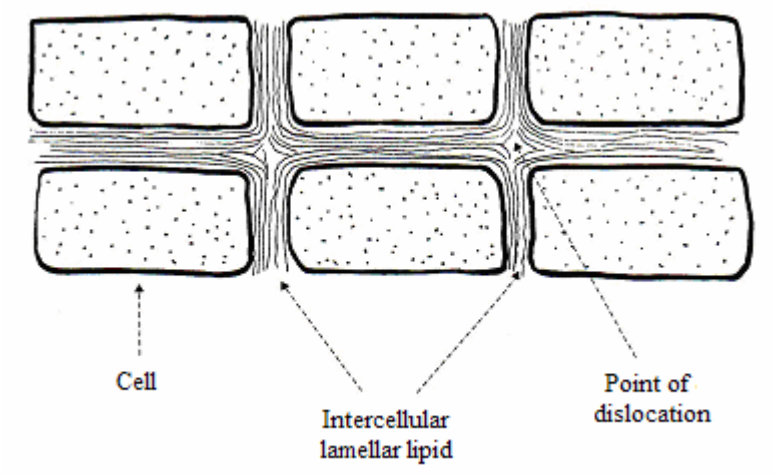


Fig. 2.3: Schematic representation of the concept of stratum corneum as a two-compartment system, in which cells can be analogized to bricks, and intercellular lamella to mortar (modified after Osborne [220]).

The actual pathway for penetration via pilosebaceous apparatus could be through the hair fibre itself, through the outer root sheath of the hair into the viable cells of the follicle, or through the air-filled canal and into the sebaceous gland [218]. The route for the sweat duct may be through either the lumen or the walls to below the epidermis and through the thin ring of keratinized cells. Dense capillary networks closely envelope the bases of both the sweat ducts and the hair follicles. Most molecules reaching these highly permeable vascular regions would immediately sweep into the systemic circulation. Researchers who favour the appendageal route as the premier mode of entry cite as evidence the rapid diffusion or charged dyes through sweat ducts when they apply a potential gradient, the formation of perifollicular wheals and the preferential staining of hair follicles with dyes.

Supporters of diffusion through the unbroken stratum corneum cite the small fraction of surface area of the appendages, the evidence that varying the number of appendages does not affect the steady state permeation and the very large activation energies measured, which would be improbable if the route was the relatively rapid appendageal pathway.

Fig. 2.4 shows a schematic representation of the routes by which drugs penetrate the skin, as well as examples of some drugs applied on the skin for particular treatments. It this diagram it is emphasized the special boundary for antifungal agents such as clotrimazole and ketoconazole once applied topically.

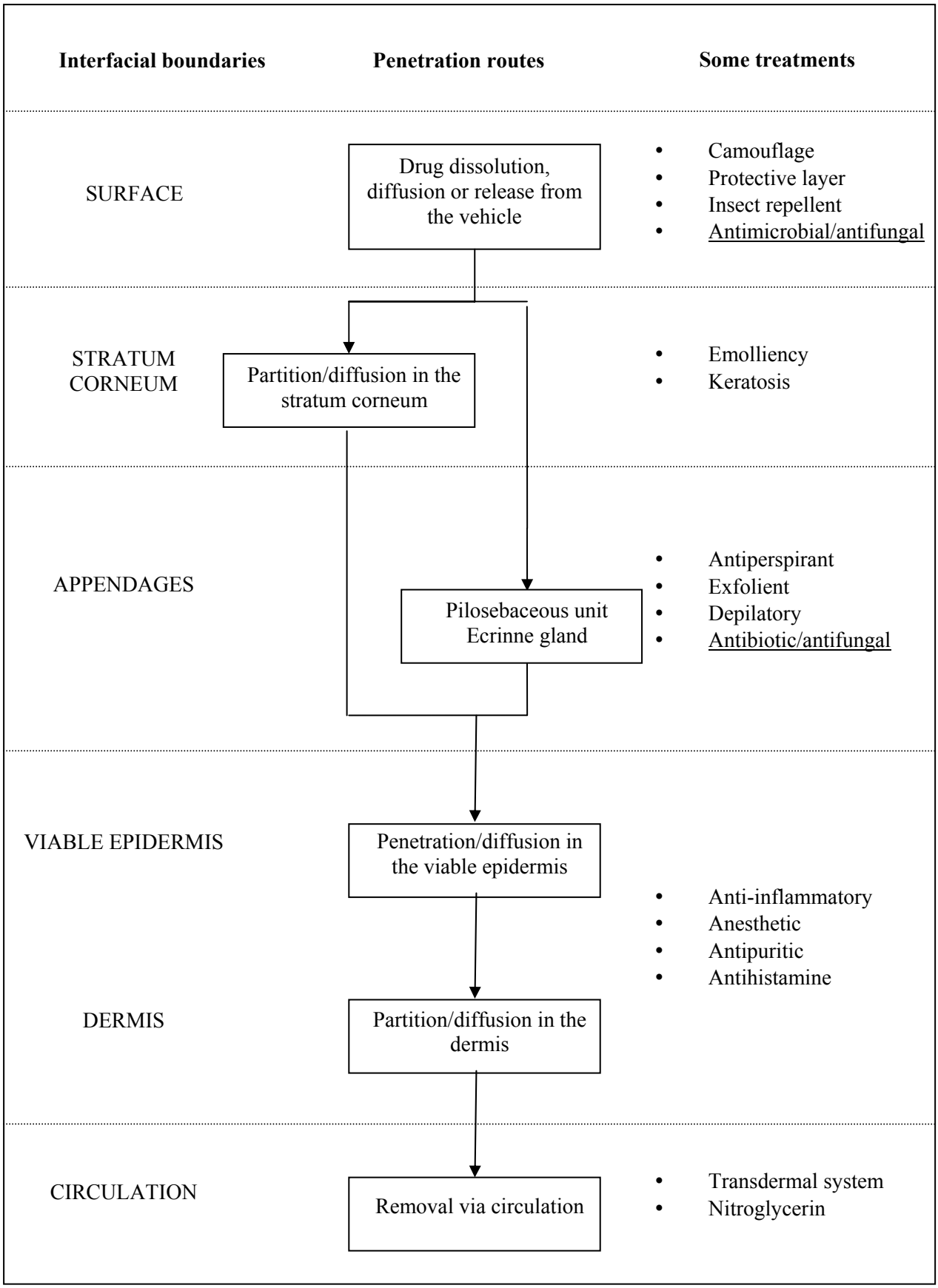


Fig. 2.4: Schematic representation of the routes by which drugs penetrate the skin (modified after Barry [218]).

2.3 Drug delivery from topical formulations

Drug delivery from topical formulations for local or systemic effects essentially involves passive diffusion of the drug through the skin. The release of a drug molecule from a vehicle into the skin and diffusion across the skin are controlled by physicochemical factors sensitive to the molecular properties of the drug molecule, the vehicle, and the skin. Rate influencing interactions are possible between the drug and skin, vehicle and skin, drug and vehicle, and drug, vehicle and skin. Examples of drug-skin interaction include changes of the surface structure of the skin by the drug components of the formulations, as well as the binding of drugs to constituents of the skin as they diffuse through the tissue field. A vehicle-skin interaction might occur when some of the vehicle's components influence the physical state of the skin, in turn, affecting the skin's permeability. Examples of these interactions are penetration enhancement effects brought about by direct solvent action on the skin, the general influence of the vehicle on the state of the skin's hydration, and vehicle effects on the local vasculature, which alter drug clearance and skin temperature, in either case, subtly influencing permeability.

Drug-vehicle interactions are those in which physicochemical interactions between the drug and the vehicle kinetically or thermodynamically govern the release of the drug into the skin. Such interactions can become the rate controlling factors and be clinically highly important when the stratum corneum is impaired as the consequence of disease or injury, for example when the skin is damaged, solution and diffusion in the vehicle may be relatively slow with regard to the skin permeation.

In the present work, it has been mainly considered the solubility of the drug and its diffusive mobility within the vehicle, as each is a factor influencing the rate of presentation of the drug to the vehicle-skin interface. For the more usual case, in which the permeability of the intact stratum corneum is low, partitioning of the drug into the skin can still exert a profound, if not dominant, influence on the rate of the delivery.

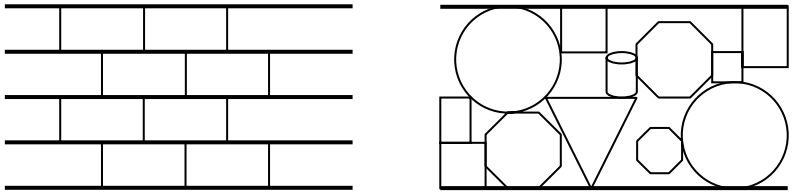
2.4 Innovative solid lipid carriers for topical drug delivery

As exposed in the chapter 1 of the present thesis, SLN and NLC are considered as the newly innovative drug carriers presenting many interesting features in the pharmaceutical field. Although brief, the foregoing description of the above mentioned lipid nanoparticles will

provide the necessary background for the discussion of the use of those carriers in topical delivery of imidazole antifungals.

2.4.1 Morphological description of SLN and NLC

Fig. 2.5 compares the lipid matrix between SLN and NLC emphasizing the differences of a perfect crystal in SLN and of a structure with imperfections in NLC.



SLN matrix **NLC matrix**

Fig. 2.5: Differences between a perfect crystal in SLN in comparison to a structure with imperfections in NLC. Perfect crystal in SLN comparable with a brick wall and structure with imperfections due to spacially very different molecules in NLC (modified after Müller *et al*. [16]).

For the production of these carriers (section 2.4.2.), the literature describes three main technologies, which are the HPH technique [10], the preparation via microemulsions technique [11] and the solvent emulsification-evaporation technique [221, 222]. A number of other methodologies used to prepare colloidal carrier systems may be encountered, once they have also been recently tested to prepare SLN. These include the solvent displacement and the emulsification-diffusion techniques, which have been used to prepare polymeric nanoparticles [59]. As expected, the production method is highly responsible for the morphological structure of lipid nanoparticles. Since the main differences between the matrices of SLN and NLC have been highlighted in the chapter 1, in the following section, the morphological differences of drug incorporation in both systems will be explained in a systematic point of view.

2.4.1.1 Models of drug incorporation into SLN

For the incorporation of drugs into SLN the literature refers three different models, according to Mehnert *et al*. [96]: (i) the homogeneous matrix model, (ii) the drug-enriched shell model and (iii) the drug-enriched core model. The morphological differences between those models

are shown in Fig. 2.6, and they depend mainly on the composition of the formulation itself, i.e. the chemical nature of the active compound, lipid and surfactant, as well as on the production procedure.

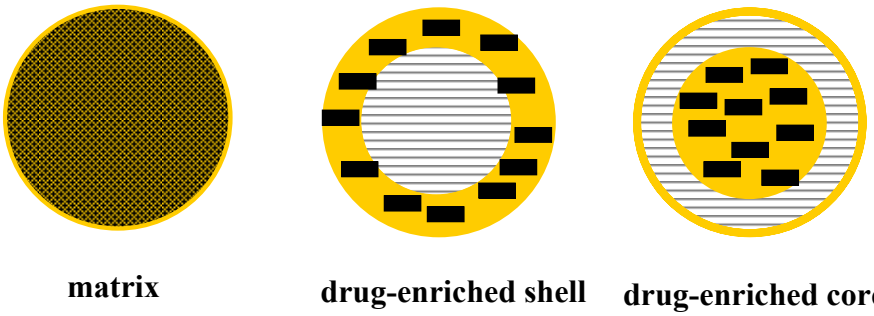


Fig. 2.6: Models of incorporation of active compounds into SLN. Black squares represent drug molecules (modified after Müller *et al.* [12]).

The matrix model (Fig. 2.6, left) is defined as a homogeneous lipid matrix with molecularily dispersed drug or drug being present in amorphous clusters. It is described for SLN prepared by the cold HPH technique, or when incorporating very lipophilic drugs in SLN when applying the hot HPH technique. When using the cold HPH, the bulk lipid contains the dissolved drug in molecularly dispersed form, where mechanical breaking by HPH leads to nanoparticles having the homogeneous matrix structure. The same will happen when the oil droplets produced by the hot HPH are cooled, crystallize and no phase separation between lipid and drug occurs. This model is assumed for entrapped drugs that can show prolonged release from SLN [12].

The drug-enriched shell model (Fig. 2.6, middle) is described by an outer shell enriched with active compound that covers a lipid core. This model is obtained when phase separation occurs during the cooling process from the liquid oil droplet to the formation of SLN when applying the hot HPH. The lipid precipitates first forming a practically compound-free lipid core. At the same time, the concentration of active compound in the remaining liquid lipid increases continuously. Finally, the compound-enriched shell crystallizes. Once the drug molecules are in contact to the outer phase of the aqueous dispersion, this model is assumed for drugs that release very fast from SLN. A fast release can be highly desired when application of SLN to the skin should increase the drug penetration, especially when using the occlusive effect of SLN at the same time [16].

The drug-enriched core model (Fig. 2.6, right) is formed when occurs the opposite mechanism described for the drug-enriched shell model. In the core model the active compound

precipitates first and the lipid shell formed around this core will have distinctly less drug. This leads to a membrane controlled release governed by the Fick's law of diffusion [16]. This model is formed when the drug concentration is close to its saturation solubility in the lipid.
These three models of incorporation of active compounds into SLN described above represent ideal types. However, there are also mixed types which can be considered as a fourth type. It has also been reported that drugs can also be linked to the outer layer of SLN composed of phospholipids and steric stabilizers [176]. Distribution of the drug depends both on its physicochemical characteristics and on the components of the SLN. However, it is most influenced by the partition coefficient of the drug molecules [175, 177].

2.4.1.2 Models of drug incorporation into NLC

With regard to NLC, the literature also describes three main models for drug incorporation, which are illustrated in Fig. 2.7.

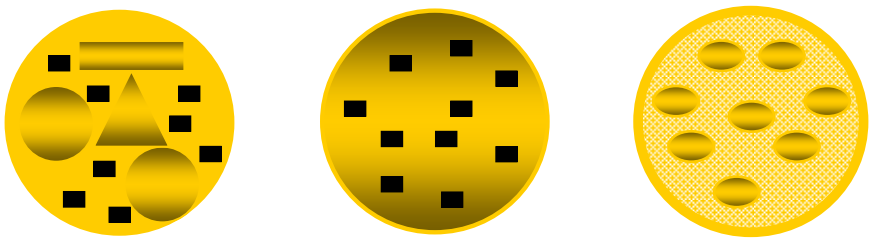


Fig. 2.7: The three types of NLC. Black squares represent drug molecules (modified after Müller *et al.* [16]).

NLC type I is defined as the "imperfect crystal type" (Fig. 2.7, left), once in its matrix there are many imperfections which are able to accommodate the drug molecules. This model is obtained when mixing solid lipids with small amounts of liquid lipids (oils). Due to the different chain lengths of the fatty acids and the mixture of mono-, di- and triacylglycerols, the matrix of NLC is not able to form a highly ordered structure [16].
NLC type II is called the "amorphous type" (Fig. 2.7, middle), and it is created when mixing special lipids which do not recrystallize anymore after homogenization and cooling processes, such as hydroxyoctacosanylhydroxy stearate and isopropyl myristate. These lipids are able to create solid particles of amorphous lipid structure, which can avoid the occurrence of

crystallization, minimizing drug expulsion once the matrix is maintained in the polymorphic α modification.

NLC type III is described as "multiple type" (Fig. 2.7, right). For a number of drugs, the solubility in liquid lipids is higher than in solid lipids [156, 160]. Therefore, the loading capacity for these drugs has been improved by the development of this model. This type is derived from water-in-oil-in-water (w/o/w) emulsions, which consists of an oil-in-fat-in-water dispersion. In fact, tiny oil nanocompartments are created inside the solid lipid matrix of the lipid nanoparticles generated by a phase separation process [16]. This model is obtained when mixing solid lipids with liquid lipids (oils) in such a ratio that the solubility of the oil molecules in the solid lipid is exceeded. A melted lipid and a hot oil are blended, and thus, the two lipids must show a miscibility gap at the used concentrations, at approximately 40^{o}C. A hot o/w nanoemulsion is produced at higher temperature (around 80^{o}C), the lipid droplets are cooled, when reaching the miscibility gap, the oil precipitates forming tiny oil droplets in the melted solid lipid. Subsequent solidification of the solid lipid as solid nanoparticle matrix leads to fixation of the oily nanocompartments. This is a procedure used to increase the solubility of a number of drugs in the particles [160].

2.4.2 Description of the preparation methods of SLN and NLC

2.4.2.1 High pressure homogenization technique

The preparation of lipid nanoparticles applying the HPH technique has been developed by Müller and Lucks [10]. In order to achieve a narrow particle size distribution, which increases the physical stability of the aqueous dispersion, a homogeneous distribution of the power density is necessary. Otherwise, particles localized at different positions in the dispersion volume will experience different dispersing forces and therefore, the degree of particle disruption will vary within the sample volume. The dispersion grade depends on the power density and the power distribution in the dispersion volume [223]. The power density (P_v) is defined as the energy (W) dissipated in the homogenization volume (V) related to the time (t), and it can be described applying the following equation:

$$P_v = \frac{W}{tV} \qquad (2)$$

Therefore, the factors determining the power density are the homogenization pressure and the width of the homogenization gap. High power densities such as $10^{12} – 10^{13}$ W/m^3 reached by high pressure homogenizers result in more effective particle disruption.

The literature describes the production of lipid nanoparticles using high shear homogenization [36, 113, 134, 153, 173, 175-177, 224-226] or ultrasounds [4, 6, 64, 107, 117, 134, 193, 207, 208]. However, it is believed that when applying high-shear homogenizers and ultra-sonication, inhomogeneous power distributions are most likely to occur. On the contrary, high pressure homogenizers are characterized by a homogeneous power distribution due to the small size of the homogenizing gap (10 – 30 μm).

This technique also overcomes a major problem of many other production procedures, which is the lack of large scale production technology. Since the fifties of the last century, HPH is a well established technology for the production of emulsions for parenteral nutrition, such as Intralipid® and Lipofundin® [9], and it can also be adapted for scale-up production of lipid nanoparticles. For HPH a good manufacturing practices (GMP) unit for clinical batch production was developed and validated which can achieve batch sizes between 2 and 10 kg of particles dispersion [227]. Further, a large scale production line was designed having a capacity of 50-150 kg of particle dispersion per hr by placing two homogenizers in series. The production of lipid nanoparticles with these lines can be performed in discontinuous or continuous modes [96, 168, 213, 228, 229].

For the homogenization process a piston-gap homogenizer or a jet-stream homogenizer (microfluidizer type) can be used. For several technical reasons, such as temperature control, cost of large scale equipment, and availability in industry, the piston-gap homogenizers are typically preferred. Examples are the equipments from APV Gaulin and from Avestin.

Lab scale production can be performed using a Micron LAB40 (APV, batch size 40 ml or for even smaller volumes) [12, 13, 228], an Emulsiflex EF-B3 or Avestin B3 (minimum batch size of approximately 3.5 ml) [165, 167, 230] or an Emulsiflex C5 [99, 100]. For larger volumes the continuous version of the LAB40 can be applied being equipped with product containers having a capacity of up to 500 ml (minimum volume of approximately 200 ml due to the dead volume of 50 ml). The LAB60 has a homogenization capacity of 60 l/hr and can be used for first technical batches. The LAB60 can be purchased with fitted product containers of up to 10 l (approximately 10 kg dispersion). The minimum batch size in the continuous mode by circulating the product is about 2 kg. The maximum batch size is 10 kg when running the LAB60 in the discontinuous mode, which means passing the product three times through the homogenizer. The production times are 10 min and 20 min, respectively

(homogenization time). The Gaulin 5.5 has been successfully employed for large scale production, having a homogenization capacity of 150 l/hr. Running a product with only one homogenization cycle will lead to about half a ton of particle dispersion within 3 hr homogenization time. Larger machines are the Rannie 118 with up to 2 tons/hr (APV systems) or the Avestin 1000 with the capacity of 1 ton/hr (Avestin). Scaling up is easily possible when moving from 40 g dispersion (Micron LAB40) to e.g. 40 kg batches produced with the Gaulin 5.5 (scalling up factor 1000). Running products on the larger machines leads even to a better product quality, i.e. more homogeneous size distribution and smaller particle sizes in less homogenization cycles. The obtained highest quality is due to the fact that larger machines are much better temperature controlled. In contrast to the LAB40, larges machines possess two homogenization valves in series and the valve design and geometry is identical or at least very similar. In addition, the larger machines are multi-plunger equipped, avoiding or minimizing pulsating of the homogenization pressure as it occurs with the one-plunger homogenizer (e.g. LAB40).

HPH can be performed using either the hot or the cold homogenization process. In both processes the active compound is dissolved or dispersed in the melted lipid prior to the HPH [9, 12]. Both techniques are suitable for processing lipid concentrations of up to 40% and generally yield very narrow particle size distributions. The polydispersity index (PI) usually obtained is lower than 0.2 [231, 232].

2.4.2.1.1 Hot high pressure homogenization

Fig. 2.8 describes the schematic procedure for the preparation of lipid nanoparticles by hot HPH technique. The active compound-containing melted lipid (1) is dispersed in the hot surfactant solution (2) at the same temperature applying high-speed stirring (3) [226, 233, 234] or alternatively an ultrasound procedure (4) [63, 133, 134, 169, 207, 226, 233-235]. These procedures involve the break-up of large droplets into smaller ones [226]. The obtained pre-emulsion (5) is passed through a high pressure homogenizer (6). The number of homogenization cycles ranges usually between 3 and 5, applying most of the times a pressure ranging from 500 bar [12, 223] to 1000-1500 bar [122, 129]. An aqueous dispersion of lipid nanoparticles is formed by the following cooling of the obtained nanoemulsion, when the lipid phase solidifies at room temperature or at temperatures below (7). Higher homogenization pressures are not necessary because in this case the material that is being processed is of lipid nature. The amount of energy introduced to the product should not be higher than the required to achieve the desired effects (nanometer range), as the increasing

pressure will result in an increase of the costs for the energy (proportional) but also for maintenance (exponential). In many cases it might be more profitable increasing the number of homogenization cycles at a moderate pressure level rather than applying one or two passes at a higher pressure [236]. This will also avoid the contamination of the product with heavy metals from the homogenizer.

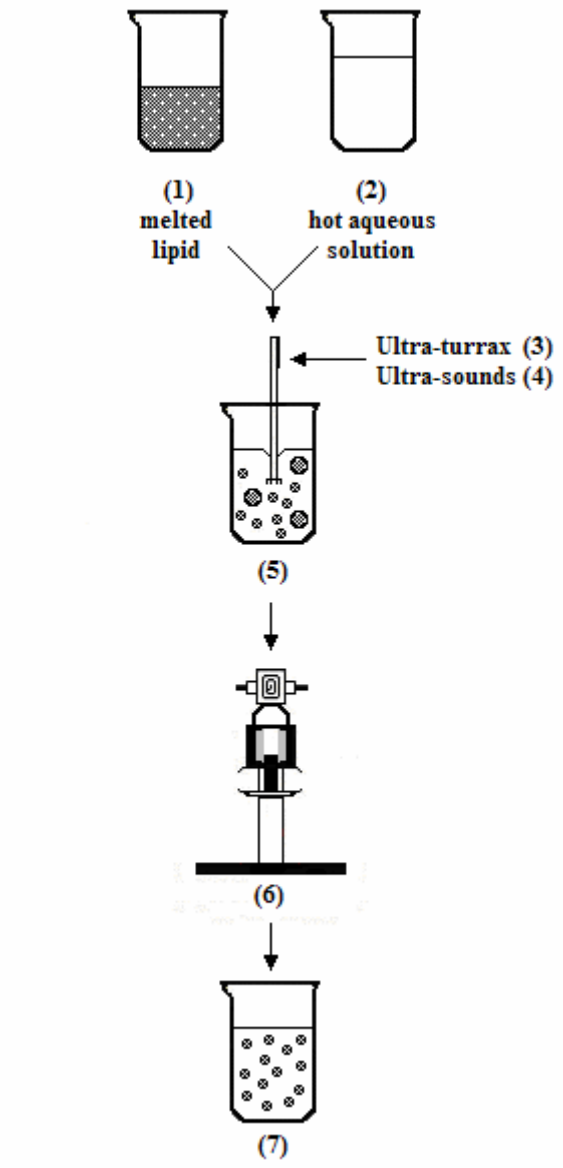


Fig. 2.8: Schematic representation of the production of lipid nanoparticles by hot HPH technique.

Hot HPH technique is the most frequently applied. It can be used for the entrapment of lipophilic and insoluble drugs in the lipid matrix. Once the exposure time to high temperatures is relatively short, in general, even temperature sensitive compounds can be processed by hot HPH, such as retinol [156] and ketoconazole [127]. However, for

hydrophilic drugs this procedure is not the most appropriated [12]. During the homogenization of the melted lipid phase the drug will partition to the water phase resulting in a too low encapsulation rate.

2.4.2.1.2 Cold high pressure homogenization

In the cold HPH technique (Fig. 2.9), the active compound-containing melted lipid (1) is cooled down by means of dry ice or liquid nitrogen (2), in order to increase the brittleness of the lipid and to ease the further milling procedure. The high cooling rate favours a homogeneous distribution of the drug within the lipid matrix [223]. After solidification, the lipid mass is ground using ball or mortar milling (3) [94, 152, 184] to yield lipid microparticles in a range between 50 and 100 μm (4) [12]. The lipid microparticles are then suspended in cold surfactant solution by stirring, yielding a macro-suspension (5). This suspension is passed through a high pressure homogenizer at/or below room temperature (6) and the microparticles are broken down to nanoparticles (7). The cavitation and shear forces in the homogenization gap are sufficiently high to break the microparticles and to reach the nanometer range. These particles stay in their solid state. Low temperatures increase the fragility of the lipid and favour, therefore, particle disruption [223].

The cold HPH technique minimizes the thermal exposure of the sample, but does not avoid it completely due to the melting of the lipid in the initial step of the process. Therefore, this technique is recommended for extremely temperature sensitive compounds and hydrophilic compounds, which might partition from the liquid lipid phase to the water phase during the hot HPH. To further minimize the loss of hydrophilic compounds to the aqueous phase of the suspension, water can be replaced by liquids with low solubility for the drug, such as oils and polyethylene glycols (PEG 600 or PEG 800) of low molecular weight (MW) [12].

Lipid particles prepared using the cold HPH technique possess a slightly higher PI and mean particle size compared to the ones obtained by hot HPH technique, using the same lipid at identical homogenization parameters (pressure, temperature and number of homogenization cycles). To further reduce the particle size and to minimize the polydispersity, a higher number of homogenization cycles can be applied [93].

The cold HPH technique can also be employed when the lipid matrix is composed by lipids with high melting points [151]. This technique is less effective in dispersing the lipids. During the production process, the lipid matrix remains mainly in the solid state despite of possible high (but short) temperature peaks occurring in the high pressure homogenizer. The

homogenization can be performed slightly below the melting point of the lipid (e.g. 5 to 10ºC) which seems to lead to a softening of the lipid during the homogenization process. The softened lipid can be more easily dispersed leading to a more uniform product of smaller mean particle size. The homogenization temperature needs to be carefully selected because otherwise the loss of hydrophilic drugs to the water phase might be too high.

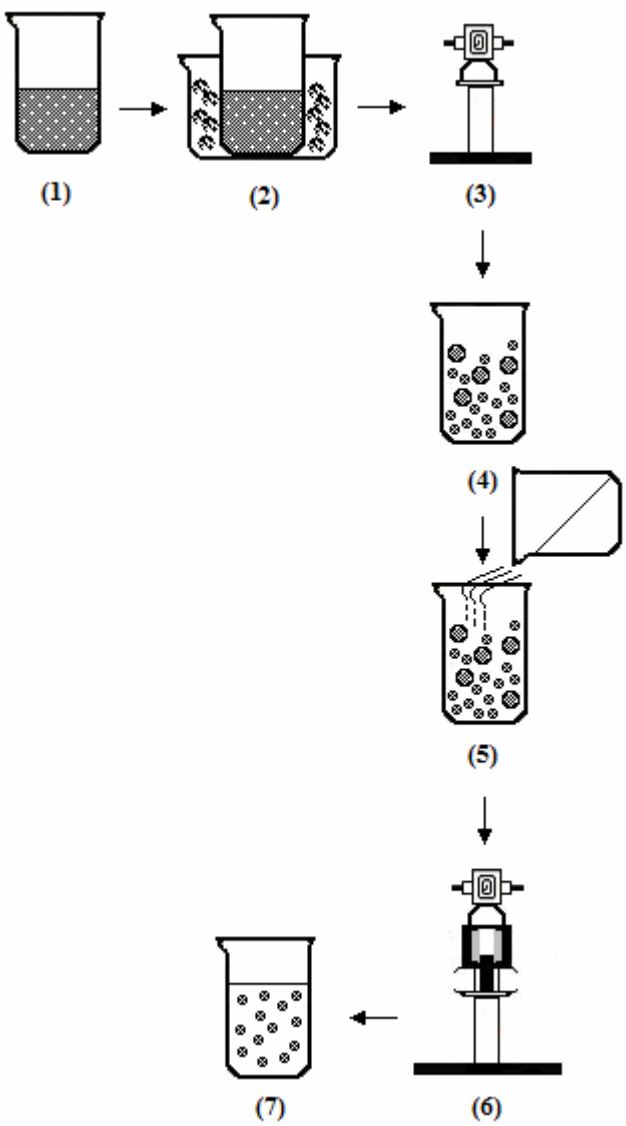


Fig. 2.9: Schematic representation of the production of lipid nanoparticles by cold HPH technique.

2.4.2.2 Microemulsions based SLN

The microemulsion technique was developed by Gasco [103] and, in the meantime it has been adapted or modified by different research groups [132, 153]. It is schematically represented in Fig. 2.10. Briefly, the lipid material is melted (1) and an o/w surfactant/co-surfactant containing aqueous phase is prepared at the same temperature (2), approximately 60-70ºC [25, 53, 56, 76, 87, 89, 103, 108, 114, 141, 146, 180, 197-199, 237]. Both lipid and aqueous phases are mixed (3) in such a ratio (typical ratios vary from 1:2 to 1:100) that a microemulsion results (4) [146]. An especially thermostatic syringe can be used [237]. Once the size of the microemulsion region in the phase diagram is a function of temperature, the system needs to be kept at elevated temperatures during the production process, in order to avoid its conversion to a different system by reducing the temperature. The hot microemulsion is then diluted into excess of cold water (5) [76, 79, 87, 89, 92, 103, 108, 114, 141, 197-199, 226]. This procedure leads to a breaking of the microemulsion, converting it into an ultra-fine nanoemulsion, which recrystallizes the internal lipid phase forming therefore the lipid particles. The dilution step can be operated at constant speed using a syringe with a needle gaugle [238].

Reasons for the breaking of the microemulsion are the dilution with water and the reduction in temperature narrowing the microemulsion region. A typical microemulsion composition is 10-15% lipid, 15-25% surfactant, 2-10% co-surfactant and 50-73% water [146]. The influence of experimental factors, such as microemulsion composition, dispersing device for adding the microemulsion to the cold water, temperature and lyophilization, on size and structure of the lipid nanoparticles has been studied intensively [146, 237, 239].

One disadvantage of the microemulsion technique is the dilution of the particles suspension with water. Typically, the concentrations are distinctly below 1% of particle content and, therefore, in case of processing to a final dosage form, large amounts of water need to be removed. In addition, high concentrations of surfactants and co-surfactants, such as butanol [76, 89, 108, 114, 144, 146, 180, 197, 237, 238, 240], or bile salts [53, 56], are necessary for stabilizing the formulation but are less desirable with respect to regulatory aspects and application. The removal of surfactants can further be performed using ultrafiltration, ultracentrifugation or dialysis [238].

For the scale-up production of lipid nanoparticles via microemulsions, a system has been developed allowing the production of 100 ml microemulsion, which is poured in excess of cold water forming lipid nanoparticles. The dispersing water ratio ranges from 1:1 to 1:10,

leading to batch sizes of up to 1.1-1. Marengo *et al*. developed a special device for scale-up production of lipid nanoparticles using this methodology [241, 242]. A schematic representation of such device is shown in Fig. 2.11. Parameters that have been investigated were the pressure of the pneumatic cylinder, the temperature and the needle gauge of the syringe containing the microemulsion, as well as the volume of dispersing water [241].

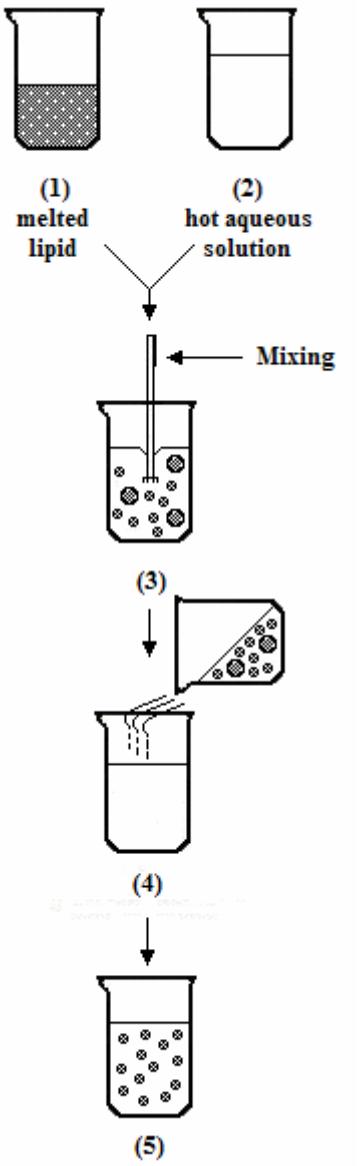


Fig. 2.10: Schematic representation of the production of lipid nanoparticles via microemulsions.

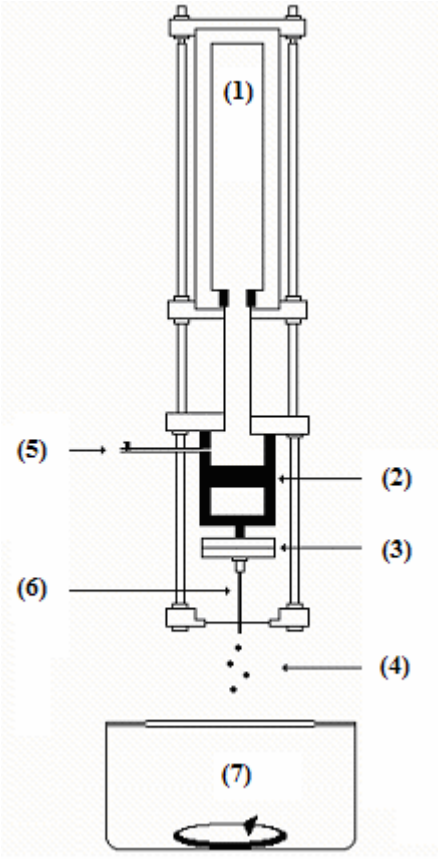


Fig. 2.11: Schematic representation of the scale up production of lipid nanoparticles via microemulsions (modified after Marengo *et al.* [241]). (1) Aluminium chamber with the hot microemulsion; (2) Thermostated wall; (3) Filter membrane of 0.22 µm; (4) Microemulsion droplets; (5) Valve; (6) Thermostated needle; (7) Cold aqueous solution.

2.4.2.3 Solvent emulsification-evaporation technique

Sjöström and Bergenståhl described a production method for preparing SLN dispersions by solvent evaporation in o/w emulsions [49]. This procedure is schematically shown in Fig. 2.12.

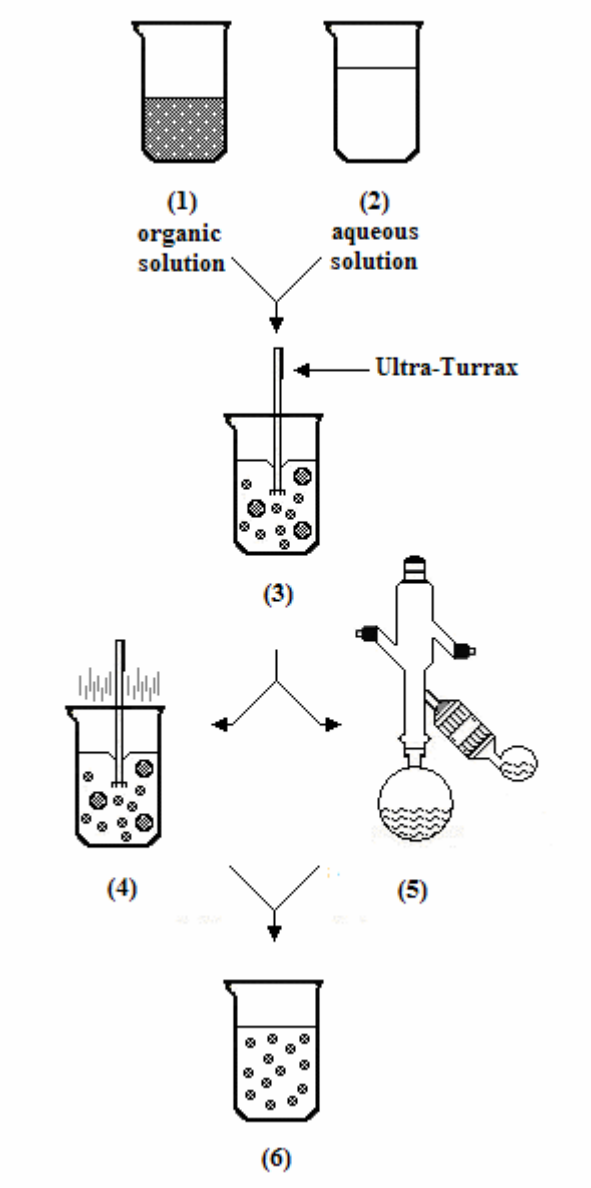


Fig. 2.12: Schematic representation of the production of lipid nanoparticles by solvent emulsification-evaporation technique.

Firstly, the lipid material is dissolved in a water-immiscible organic solvent, such as cyclohexane [49, 51], chloroform [51], ethyl acetate [153] or methylene chloride [39, 40, 124, 172, 182], and then the drug is dissolved or dispersed in that solution, producing an organic phase containing drug (1). This organic phase is emulsified in an o/w surfactant containing

aqueous phase (2) by mechanical stirring (3). Upon evaporation of the organic solvent from the obtained o/w emulsion under mechanical stirring (4) [172] or reduced pressure (5) [49, 51], a nanoparticle dispersion is formed by precipitation of the lipid in the aqueous medium (6). The solvent evaporation step must be quickly in order to avoid particle aggregation [12]. This is a method analogous to the production of polymeric nanoparticles and microparticles by solvent evaporation [51]. It can be applied for the incorporation of hydrophilic molecules such as peptides and proteins, which must be previously dissolved in a water phase preparing in this case a w/o/w emulsion [39, 40, 124, 182].

The clear advantage of this procedure over the cold HPH technique described by Müller *et al*. [10], is the avoidance of any thermal stress. The major disadvantage is the use of organic solvents [223], which are related to the increase of toxicity of the final product [12, 223].

Sjöström *et al*. have calculated the amount of toluene residues as 20-100 ppm in final dispersions. These dispersions are usually very dilute, because of the limited solubility of lipid in the organic material. Typically, lipid concentrations in the final dispersion range around 0.1 g/l, therefore, the particle concentration has to be increased by means of e.g. ultra-filtration or evaporation [221, 222].

2.4.2.4 Solvent displacement technique

The solvent displacement technique was first described by Fessi *et al*. [243] for the preparation of polymeric nanoparticles from pre-formed polymers. Recently, this technique has also been used to prepare SLN [54, 65, 66, 109, 244]. Fig. 2.13 describes schematically this production procedure.

In this process, the lipid material is dissolved in a semi-polar water-miscible solvent, such as ethanol, acetone or methanol [54, 65, 109, 244], and then the active compound is dissolved or dispersed in this phase (1). Simultaneously, an o/w surfactant containing aqueous phase is prepared (2). The organic phase is injected into the aqueous phase under magnetic stirring (3). A violent spreading is observed because of the miscibility of both phases. Droplets of solvent of nanometer size are torn from the o/w interface. These droplets are rapidly stabilized by the surfactant molecules that are in the aqueous phase, until diffusion of the solvent is complete and lipid precipitation has occurred. Removal of solvent can be performed by distillation (4) and the lipid nanoparticles are formed after total evaporation of the water miscible organic solvent (5).

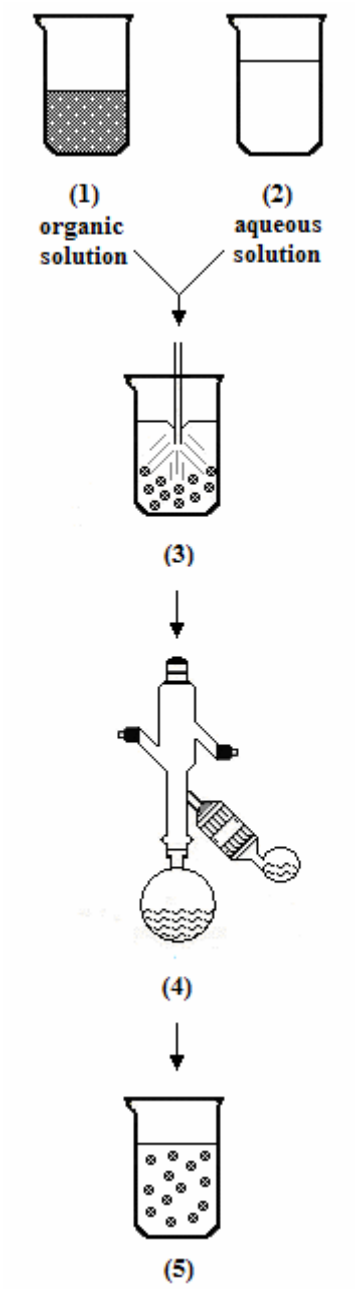


Fig. 2.13: Schematic representation of the production of lipid nanoparticles by solvent displacement technique.

2.4.2.5 Emulsification-diffusion technique

The emulsification-diffusion technique patented by Quintanar-Guerrero and Fessi in 1999 [245], is usually used to produce polymeric nanoparticles based on synthetic polymers. However, it has been recently applied to prepare lipid nanoparticles [42-44, 246, 247] and it is schematically represented in Fig. 2.14.

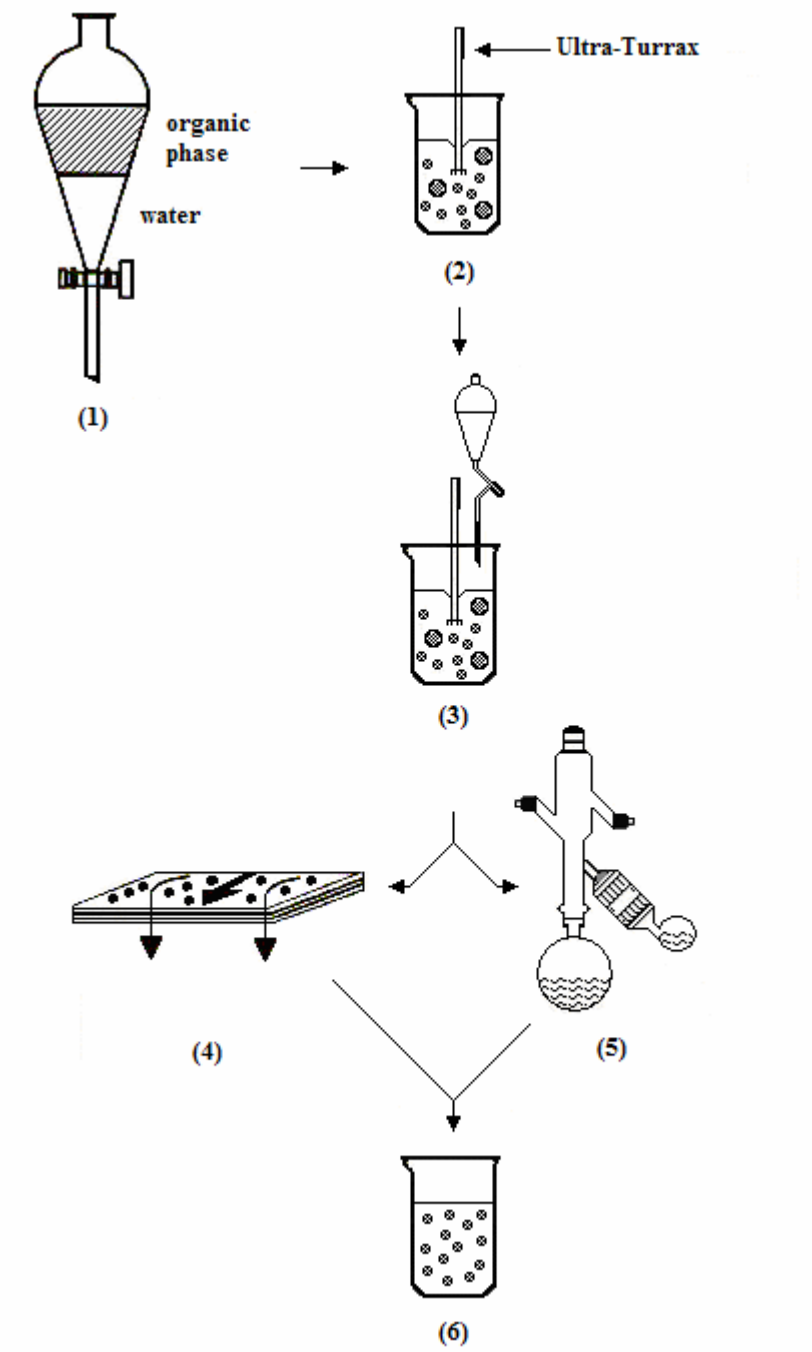


Fig. 2.14: Schematic representation of the production of lipid nanoparticles by emulsification-diffusion technique.

This procedure involves the use of a partially water soluble solvent (1), such as benzyl alcohol [246], isobutyric acid [125] or tetrahydrofuran [42-44], which is previously saturated with water to ensure the initial thermodynamic equilibrium between the two liquids (water and solvent). The lipid is dissolved in the saturated solvent producing an organic phase where the drug is added. This organic phase is then emulsified, under vigorous agitation, in an aqueous

solution containing a stabilizing agent for the preparation of an o/w emulsion (2). The subsequent addition of water to the system (3), under moderate mechanical stirring, causes solvent diffusion into the external phase and the lipid starts precipitating. Depending on its boiling point, the solvent can be eliminated by ultra-filtration (4) or by distillation (5). After the organic solvent being totally eliminated, an aqueous dispersion of lipid nanoparticles is formed (6). Average particle sizes around 100 nm and very narrow particle size distributions can be achieved by this method [44].

2.4.2.6 Phase inversion-based technique

Recently, a novel phase inversion-based technique has been described by Heurtault *et al*. [248, 249], for the preparation of lipid nanoparticles. Basically, this procedure consists of two steps, which are schematically represented in Fig. 2.15.

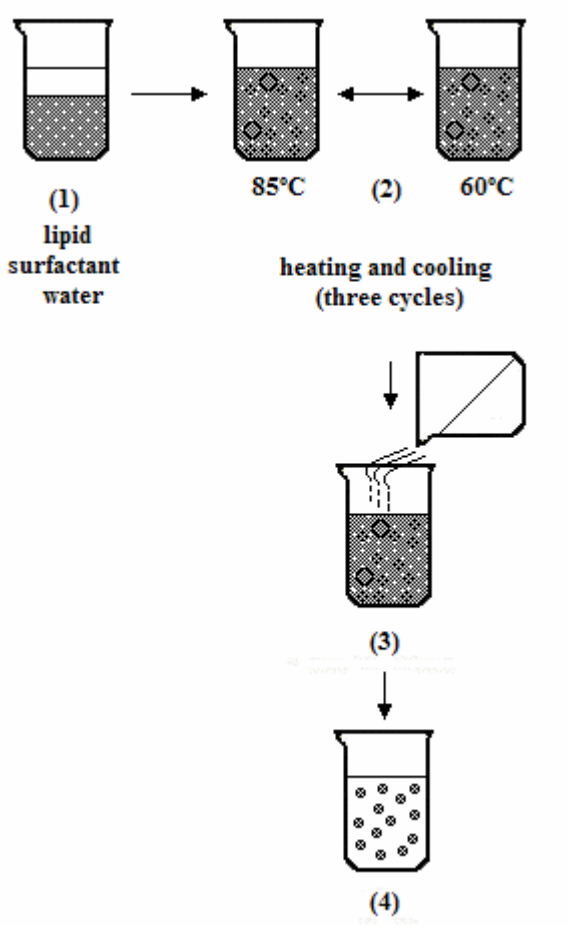


Fig. 2.15: Schematic representation of the production of lipid nanoparticles by phase inversion-based technique.

The first step consists of magnetic stirring of all the components (lipid, surfactant and water) in the correct proportions which need to be previously defined for each formulation (1). Three cycles of heating and cooling from room temperature to 85ºC and back to 60ºC are subsequently applied at a rate of 4ºC/min (2). This thermal treatment (85ºC-60ºC-85ºC-60ºC-85ºC) will reach the inversion of the emulsion. The second step consists of dilution under cooling conditions (3). The temperature of the system before the dilution can be determined at the beginning of the inversion process and it is named T_{cd} (cd stands for cooling dilution). An irreversible shock induced by dilution with cold water to the mixture maintained at the previously T_{cd} will break the system. This fast cooling dilution process with cold distilled water (approaching 0ºC), leads to lipid particles in the nanometer range (4). Afterwards, a slow magnetic stirring is applied to avoid particle aggregation.

This technique has the advantage of the possibility to modify the temperature range (and consequently the T_{cd}) with salinity, which is very important regarding the incorporation of thermolabile drugs [249]. Drug degradation is not expected to occur once the heating period is very short. In addition, this technique also avoids the use of organic solvents.

Before the cooling dilution an optimized components proportion should be done using a ternary diagram, because this influences the size parameters of the obtained particles [250, 251].

2.5 Problems of production and stability of SLN and NLC

2.5.1 Lipid polymorphism

One of the main problems associated to the lipid nanoparticles and their physicochemical stability during shelf life is the polymorphic transformations that lipid matrix can suffer. Polymorphism is, in fact, the ability of a compound to crystallize in more than one distinct crystalline species with different internal lattice structures. Lipid crystallization is an important point for the performance of the lipid nanoparticles. Therefore, polymorphism and the polymorphic transitions of acylglycerols are discussed in this section. The internal structure of the lipid matrix can occur in a variety of ways, as shown in Table II. Lipids can crystallize in different three-dimensional structures, which are represented in Table III. Concerning the organization of lipid molecules such as triacylglycerols, according to Larsson short spacings of these molecules can be described as follows:

α modification:

Hexagonal (H) subcell with a lattice spacing of 0.415 – 0.42 nm

β' modification:

Orthorhombic perpendicular ($O_{\perp}$) subcell with strong lattice spacings of 0.42 – 0.43 nm and 0.37 – 0.40 nm

β modification:

Triclin parallel ($T_{//}$) subcell with strong lattice spacings of 0.46 nm

Table II: Characterization of the crystal systems (modified after Barrow [252]).

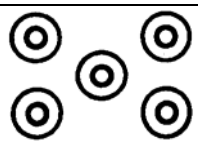
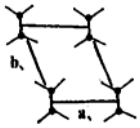
Acyl chain orientation	**Lattice**	
	Axle	*Angle*
Cubic	a = b = c	α = β = γ = 90°
Hexagonal	a = b, c	α = β = 90° γ = 120°
Rhomboedric	a = b = c	α = β = γ ≠ 90°
Tetragonal	a = b, c	α = β = γ = 90°
Orthorhombic	a, b, c	α = β = γ = 90°
Monoclinic	a, b, c	α = β = 90°
Triclinic	a, b, c	α, β, γ

Further polymorphic forms are found when using complex acylglycerols, such as mixed acid triacylglycerols or partial acylglycerols. Multiple β' and β, sub-α or intermediate forms, usually mentioned as β_i, have been described. However, nomenclature and properties of monoacid triacylglycerols can also be used for these complex acylglycerols that have similar crystal packing [253].

Crystallization of bulk triacylglycerols from the melt after rapid cooling usually occurs in the less stable α modification, which transforms via the β', into the more stable β modification upon heating or during storage time. In colloidal dispersions of lipid nanoparticles, these transformations of triacylglycerols are faster than in the bulk material, which lead to a change

in the relative fraction of the polymorphic forms [2, 133]. Depending on the chemical nature of the lipid and on the production parameters, different fractions of α and β' modifications may occur. This phenomenon can lead to a reduction of the melting point, or more precisely, to changes in the form and shift of the melting peak. These created polymorphic forms are not long-term stable, leading to a gradual transformation to more stable modifications, which means increasing content of β'/β_i and finally β. This is not desired because the change in lipid structure is responsible for drug expulsion during storage and changes in the release profile of incorporated drug, as well as changes in particle size parameters.

Table III: Three-dimensional structure of the crystal order in the three main polymorphs from monoacid triacylglycerols (modified after [254, 255]).

	α modification	**β' modification**	**β modification**
Crystal system	Hexagonal	Monoclin	Triclin
Subcell	Orthorhombic	Orthorhombic	Triclin
b, a			
c, b			
c, a			

Drug expulsion from suppository lipid matrices is a very well known phenomenon since many years. There are many possibilities for incorporating drugs in the lipid matrix. In general, when a crystal is formed, foreign molecules can be incorporated by replacing host molecules in the lattice by a guest molecule or by being incorporated in between host molecules. However, the guest molecule needs to be 20% smaller than the host molecule.

In addition, drugs can be localized in between the lipid lamellae, resulting in increase of the lattice spacing "d", that can be analysed by X-ray diffraction studies. The drug can also be present in form of amorphous clusters, mainly localized in the imperfections of the crystal. In

this case, drug accommodation is improved when the lipid crystal has more imperfections. Thus, drug loading can be increased by using rather crude lipid mixtures or by controlled nanostructuring of the lipid matrix, i.e. creating as many imperfections as possible. Depending on the nature of the lipids used for blending the lipid matrix, different types of NLC can be obtained, as previously discussed in the section 2.4.

2.5.2 Risk of gelation

Aqueous SLN and NLC dispersions of low viscosity have potentially the risk of transformation into a viscous gel, resulting in loss of their colloidal size and increase of particle size, i.e. occurrence of aggregation [223]. In the majority of the situations this transformation occurs very fast and in an unpredictable way, and might be related to several factors, such as:

1. contact of the aqueous dispersion with strange surfaces such as syringe needle used during particle size measurements
2. increase of external shear forces, i.e. mechanical stress, during sample manipulation and transport
3. exposure to high temperatures, light and oxidizing atmospheres
4. formulations prepared with high lipid concentrations and high ionic strengths
5. polymorphic transformations during storage time

To avoid gel formation one can act in order to increase the physicochemical stability of the formulations. For instance, gelation can be retarded or avoided by addition of co-emulsifying surfactants with high mobility [256]. Storage at temperatures lower than at room temperature (25ºC), under dark conditions and nitrogen atmosphere can prevent particle growth [257].
The state of crystallinity of the lipid particles is also related to their physical stability in aqueous dispersions, i.e. absence or induction of particle aggregation and finally gel formation [12, 223]. It was observed that particle aggregation was accompanied, simultaneously, by transformation of high energetic polymorphic forms (i.e. mainly α modification) to more stable lipid modifications. In general, the next step after particle aggregation is the increase of viscosity and the formation of soft and finally relatively rigid gels. In order to avoid particle aggregation one interesting approach, especially concerning the development of semi-solid formulations, is the entrapment of lipid nanoparticles in creams and hydrogels. This subject will be discussed in the chapter 5 of the present thesis.