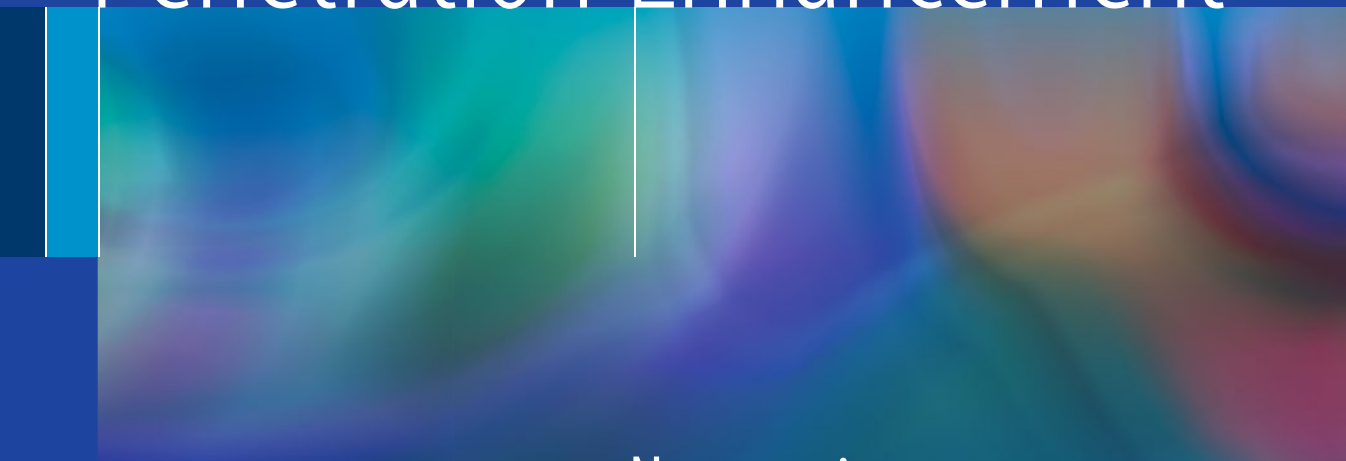
Percutaneous Penetration Enhancers

Chemical Methods in Penetration Enhancement



Nanocarriers

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Preface

The main function of skin is the protection of the body from the external environment by preventing loss of water and the ingress of exogenous substances. This implies that the skin acts as a barrier for the diffusion of substances into the underlying tissue. Despite this role, the skin has become recognized as an important drug delivery route which can be reached directly. It is an ideal site for the application of drugs for achieving local (topical) and systemic (transdermal) drug effects. Local or topical drug delivery assumes treating various skin diseases, while transdermal delivery aims to achieve systemically active drug levels in order to treat systemic diseases. Drugs have been applied to the skin to achieve also regional drug delivery which involves drug application to the skin to treat or alleviate disease symptoms in deep tissues beneath the skin (such as in musculature, etc.). Topical and transdermal drug delivery offer a number of advantages compared to other conventional routes, and hence they are of great interest to pharmaceutical research, which explains the increasing interest in skin as a site of drug application.

However, skin represents a formidable barrier for percutaneous drug absorption, being of crucial importance for achieving topical and transdermal effects of drugs. Significant efforts have been devoted to developing strategies to overcome the impermeability of intact human skin. There are many ways for circumventing the stratum corneum, which provides the main barrier to drug penetration. These methods can be divided into chemical and physical penetration enhancement methods, i.e. percutaneous penetration enhancers, which are described in this book series *Percutaneous Penetration Enhancers*.

The aim of this book series is to provide to readers working in academia and industry, including young researchers, an up-to-date comprehensive work describing all the important topics required to understand the principles of enhancing transdermal and dermal drug delivery. The book series contains five books.

The book *Chemical Methods in Penetration Enhancement: Drug Manipulation Strategies and Vehicle Effects* begins with a description of the skin, as understanding of its structure, function and especially its penetration pathways is fundamental to understanding how topical and transdermal dosage forms work and how different methods may be employed to enhance percutaneous drug penetration. The first two parts of the book devoted to skin and the stratum corneum, representing its uppermost layer being responsible for its protection, discuss their structure, the importance of the lipid organization in the stratum corneum, the different penetration pathways through the skin

with an emphasis on the increasing importance of the follicular route, as well as the influence of different excipients on the skin. The focus of the book is on the chemical methods used to overcome the impermeability of intact skin, such as different drug manipulation strategies (drug or prodrug selection, chemical potential control, eutectic systems, complexes with cyclodextrines, etc.) and formulation/vehicle effects (influences of: emulsions, nanoemulsions, pickering emulsions, microemulsions, emulsifiers, emollients, liquid crystalline structures, gels, etc.) on the penetration enhancement of drugs.

The book *Chemical Methods in Penetration Enhancement: Nanocarriers* describes similarly to the first book chemical methods used in penetration enhancement of drugs. However, this book is devoted to the application of different kinds of nanocarriers and represents an attempt to familiarize the readers with the importance of nanocarriers used to enhance the percutaneous penetration of drugs as they have numerous advantages in comparison to conventional drug formulations. More recently, different types of nanocarriers have been designed by researchers which allow controlled and targeted drug delivery (dermal or transdermal drug delivery), improved therapeutic effectiveness and reduced side effects of drugs. As carriers they can be classified into lipid-based vesicles (e.g. liposomes, transfersomes, invasomes, etc.), surfactant-based vesicles (e.g. niosomes, novasomes and others), lipid-based particulate carriers (e.g. solid lipid nanoparticles, nanostructured lipid carriers and lipid nanocapsules), polymer-based particulate carriers (e.g. polymeric nano- and microparticles, polymeric nanocapsules, polymeric micelles, dendrimers, dendritic core-multishell nanocarriers, etc.), nanocrystals and others. This book focusing on the different nanocarriers gives a comprehensive review of their use as promising dermal and transdermal drug delivery systems. It also considers the use of nanocarriers for cutaneous immunization offering the important advantage of being painless and having a stronger immune response compared to the intramuscular injection of vaccines. In addition, the book provides insights on the safety of the use of nanoparticles.

The book *Chemical Methods in Penetration Enhancement: Modification of the Stratum Corneum* similarly to the aforementioned two books describes the chemical methods used in penetration enhancement of drugs with an emphasis on the enhancing methods used to modify the stratum corneum. It starts with the classification of penetration enhancers, their mode of action and provides insights on the structure–activity relationship of chemical penetration enhancers. The focus of this book is on the most commonly used classes of skin penetration enhancers being investigated in scientific literature and used in commercial topical and transdermal formulations, and their representatives are discussed in more detail, including their mechanism of action, where known. The following penetration enhancers are considered in the book: alcohols (e.g. ethanol, etc.), glycols (e.g. propylene glycol, etc.), amides (e.g. 1-dodecylazacycloheptan-2-one or laurocapram (Azone®), etc.), fatty acids (e.g. oleic acid, etc.), fatty acid esters (e.g. isopropyl myristate, etc.), ether alcohols (e.g. diethylene glycol monoethyl ether (Transcutol®)), pyrrolidones (e.g. N-methyl-2-pyrrolidone, etc.), sulphoxides (e.g. dimethyl sulphoxide, etc.), surfactants (e.g. polysorbates, etc.), terpenes (e.g. L-menthol, etc.), peptides and new classes of enhancers, such as iminosulfuranes,

transcarbams, dimethylamino acid esters and dicarboxylic acid esters. In addition, synergistic effects of different chemical penetration enhancers have been discussed in the book as an important feature of chemical penetration enhancers. Furthermore, the safety profile of chemical penetration enhancers is considered.

The book *Physical Methods in Penetration Enhancement* considers the current status and possible future directions in the emerging area of physical methods being used as potent enhancers for the percutaneous penetration of drugs. It gives a comprehensive overview of the most used methods for enhancing dermal and transdermal drug delivery. It covers sonophoresis, iontophoresis, electroporation, magnetophoresis, microneedles, needle-free jet injectors, ablation methods (electrical, thermal or laser skin ablation) and others. The numerous advantages of these methods have opened new frontiers in the penetration enhancement of drugs for dermal and transdermal drug delivery. Cutaneous vaccination and gene delivery by physical methods have been also discussed in this volume. Consideration was given to new methods, too, such as a novel electrochemical device for penetration enhancement, different waves (e.g. photoacoustic waves, microwaves, etc.), natural submicron injectors, moxibustion and others. Furthermore, the combined use of different physical methods or of physical methods and passive enhancement methods (chemical penetration enhancement methods) are discussed as they provide, due to their synergistic effects, higher percutaneous drug penetration when used together.

The book *Drug Penetration Into/Through the Skin: Methodology and General Considerations* provides fundamental principles of the drug penetration into/through the skin, from covering basic mathematics involved in skin permeation of drugs, influences of drug application conditions and other factors on drug penetration, mechanistic studies of penetration enhancers, influences of the type of skin used (human native or reconstructed skin) to different methods utilized to assess the drug penetration into/through the skin and to determine the amount of permeated drug (such as tape stripping of the stratum corneum, electron spin resonance, Raman spectroscopy, attenuated total reflection, confocal laser scanning microscopy, single and multiphoton microscopy, etc.). Retardation strategies are also discussed as being important for some classes of substances, such as sunscreens. The safety of applied penetration enhancers as well as the research ethics in the investigation of dermal and transdermal drug delivery are addressed in this book. The book ends with the current status and future perspectives of passive/chemical and active/physical penetration enhancement methods as they are gaining extensive interest as promising tools to enable an efficient dermal or transdermal drug delivery.

We are very thankful to all the authors who contributed chapters to the book series *Percutaneous Penetration Enhancers*, as they found time to work on the chapters despite having busy schedules and commitments. All the authors are eminent experts in the scientific field which was the subject of their chapter, and hence their contribution raised the value of the book. We also sincerely thank our collaborators from Springer: Ellen Blasig, Isabella Formento, Sverre Klemp, Srinath Raju, Andre Tournois, Grant Weston and

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Role of Nanotechnology in Skin Delivery of Drugs

1

Mehtab J. Abla, Neha D. Singh, and Ajay K. Banga

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1.1 Introduction

1.1.1 Skin

The skin is the largest organ of the human body accounting for more than 10 % of the body mass and covering an area of approximately 2 m^2. The skin has an integrated, complex structure, and it serves as a very effective barrier to entry of exogenous substances (Hadgraft 2001, 2004; Jain et al. 2010). The full thickness of human skin is made up of three layers: the epidermis, dermis, and hypodermis. The hypodermis is the innermost layer which acts as an insulator. Sweat and pilosebaceous glands arise from the hypodermis and open at the surface of the epidermis (stratum corneum). The dermis which lies below the epidermis is 1–3 mm thick and comprises of a network of blood capillaries, lymphatic vessels, and nerve endings (Bouwstra et al. 2003; Schafer-Korting et al. 2007). It is mostly made up of connective tissue and is more hydrophilic in nature and provides nutrients and oxygen to the skin. The epidermis is the outermost layer of the skin; it is composed of five different layers, i.e., the stratum lucidum, stratum granulosum, stratum spinosum, stratum germinativum (which together form the viable epidermis), and stratum corneum (SC, the outermost layer exposed to the environment) (Elias 1991; Lampe et al. 1983).

SC is made up of densely packed dead keratin-filled corneocytes. SC is 10–20 micron thick and

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conventionally described by the "brick and mortar" model, where "bricks" are the hexagonal corneocytes and the lipidic matrix surrounding these corneocytes is called the "mortar" (Prow et al. 2011). The stratum corneum structure is thus composed of corneocytes stacked upon each other in approximately 15 layers along with the lipidic matrix of ceramides, free fatty acids, triglycerides, and cholesterols forming a 500 micron tortuous pathway in between (Cevc 2004). Hair follicles arise from the hypodermis region, and they are surrounded by a rich network of blood capillaries. Drug penetration via the transappendageal route, especially via the hair follicles, was disregarded in the past because it was assumed that hair follicles occupy only 0.1 % of the total skin area. However, Otberg et al. performed a study where caffeine incorporated in a shampoo formulation was applied to volunteers and caffeine was detected in blood after 5 min; this quick transport was attributed to the delivery via transappendageal route (Otberg et al. 2008). Studies have shown that there is significant variation in hair follicle density across the body regions; the 0.1 % value is true for the inner side of the forearm; however, the highest average hair follicle density is found on the forehead followed by the scalp and face. The hair follicle density on the forehead is 292 follicles/cm^2 (Lademann et al. 2007; Lekki et al. 2007; Wosicka and Cal 2010).

1.1.2 Penetration Pathways

A drug molecule will permeate into the skin if it has favorable physicochemical properties such as size is less than 500 Da, is uncharged, and is moderately lipophilic, i.e., has a logarithm of partition coefficient (log P) between 1 and 3. A molecule will permeate via one of the three established pathways into/through the skin, i.e., transcellular, intercellular, and transappendageal routes. Passage of drug molecule through keratinocytes (i.e., cells in epidermis) is called the transcellular route, whereas passage via the lipid matrix is called the intercellular route. Transappendageal route is across hair follicles, sweat glands, and sebaceous glands (Banga 2011).

Delivery through skin can be classified into topical and transdermal; a topically active drug is meant to act locally, whereas transdermal route aims at systemic delivery. The advantages offered by skin delivery include bypassing hepatic metabolism, avoiding side effects associated with oral delivery of drugs which act on skin, easy removal of medication in case of overdose, sustained release of drug, and patient compliance (Banga 2011). As mentioned earlier, the robustness of the stratum corneum barrier prevents the entry of large and hydrophilic therapeutic agents through the skin. Extensive research has been conducted to enable delivery through the skin which has led to use of different methods including penetration enhancers and active enhancement techniques like iontophoresis, electroporation, phonophoresis, and microporation (Banga 1998, 2009; Denet et al. 2004; Mitragotri and Kost 2004). Chemical penetration enhancers work by disrupting the membrane structure of the skin; this has prevented their widespread approval and use due to concerns regarding long-term effects. Similarly, active techniques require special considerations such as fabrication of microneedles, and electrical device in case of iontophoresis may need separate FDA approval process for devices. Furthermore, acceptance of patients to the new technology, addressing concerns like sterility of microneedles, safety of an electrical device, and standardized application method in different clinical settings need to be considered. On the other hand, feasible changes to existing passive techniques which allow transport of more drug candidates through the skin can offer a good alternative. Nanotechnology to formulate drugs is one such approach which has been studied. Different routes of drug delivery, i.e., oral, parenteral, and nasal, have explored this technology for improving/enabling drug transport which emphasizes the growing popularity of nanotechnology in pharmaceutical industry. This chapter will discuss how nanotechnology can be applied for delivery through the skin, including details of various nano-carriers, their significance, and their role in enabling better penetration (Fig. 1.1).

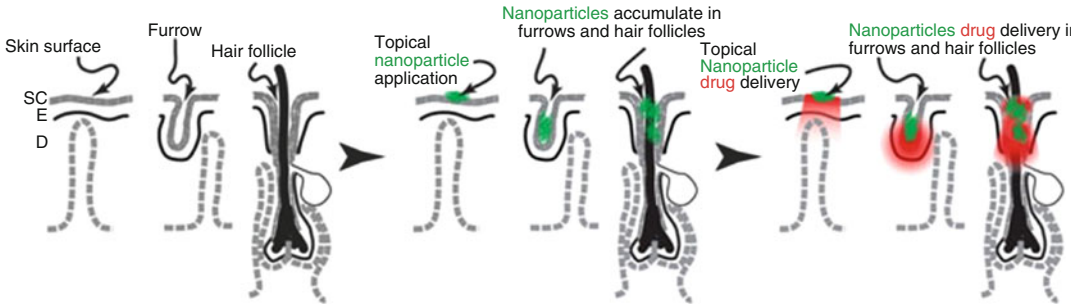

Fig. 1.1 Schematic showing penetration pathways of nanoparticles and drug delivery into skin

1.2 Nanotechnology

Nanotechnology refers to use of particles in nano-size range, typically ranging from less than 100 to 1000 nm (Baroli 2010; Escobar-Chavez et al. 2012; Mishra et al. 2010; Muller-Goymann 2004). Nano-carriers have been used for passive delivery of drugs into and through the skin since they offer several advantages over conventional passive delivery like increased surface area, higher solubility, site-targeted delivery, improved stability, controlled release of active ingredient, reduced skin irritancy, protection from degradation, increased drug loading, and improved permeation of active ingredient into the skin (Abramovits et al. 2010; Albert et al. 2012; Escobar-Chavez et al. 2012). Various research groups and pharmaceutical industry have conducted studies for developing nano-carriers suitable for skin delivery (Alvarez-Roman et al. 2004b; Mishra et al. 2009; Muller et al. 2000; Verma et al. 2003). However, the largest impact of nanotechnology on skin delivery has perhaps been through cosmetics industry; this is evident from the nano-carriers inventions and patents registered/issued and the growing list of such products in the consumer market. The main mechanism which makes these carriers more effective than currently available formulations is the size of nanoparticles; this parameter also determines the efficacy and targeted site of delivery. Passive permeation of nano-carriers larger than 20 nm through transcellular route is highly unlikely due to the densely packed nature of keratinocytes; however, small nano-carriers less than 5–7 nm can permeate into the stratum corneum. Transappendageal route has been widely investigated for delivery of nano-carriers; nano-carriers larger than 20 nm but less than 200 nm can penetrate deeply into the hair follicle via the hair opening (Albert et al. 2012; Baroli 2010; Desai et al. 2010). Shim et al. conducted a permeation study of polymeric nano-carriers loaded with minoxidil and concluded that as the particle size decreases, permeation of these nano-carriers increases (Shim et al. 2004). Nano-carriers can deliver both hydrophilic and lipophilic molecules (Abramovits et al. 2010; Kuchler et al. 2009a, b). Apart from small molecules, protein molecules which are hydrophilic macromolecules have also been delivered using nano-carriers (Choi et al. 2012; Rastogi et al. 2009). Chitosan-conjugated Pluronic® F127-based (poloxamers – triblock copolymer) (BASF Corp., Seoul, Korea) flexible nanospheres were designed for transcutaneous delivery of proteins having different molecular weights (insulin and bovine serum albumin tagged with fluorescent marker fluorescein isothiocyanate, FITC). The nanospheres allowed efficient loading of hydrophilic substituent in addition to being deformable, and penetration-enhancing properties of chitosan lead to desired delivery profile (Choi et al. 2012). Advent of imaging technology has helped to visually observe nano-carriers delivered in the skin for studying their penetration pathway and mechanism. Imaging techniques such as confocal imaging, fluorescence microscopy, electron paramagnetic resonance spectroscopy, ion microscopy, and autoradiography have been reported to visualize nano-carriers in the stratum

corneum and other layers of the skin (Campbell et al. 2012; Haag et al. 2011a, b; Lademann et al. 2007; Lekki et al. 2007; Wosicka and Cal 2010).

1.2.1 Lipid Nano-carriers

Liposomes are the first class of nano-carriers developed in the early 1970s for drug delivery system. They can be compared to biological membrane as they are made up of phospholipids and cholesterol (El Maghraby et al. 2006, 2008). Liposomes are used to transport hydrophilic as well as lipophilic drug molecules and are non-toxic in nature. Charge on liposome depends on the functional group and pH of the medium. Liposomes can be formulated in sizes ranging from 25 nm to a few microns depending on the method of preparation, process and excipients selected for the manufacture. Liposomes are classified as small unilamellar vesicles (SUV), large unilamellar vesicles (LUV), and multilamellar vesicles (MLV) based on vesicle size and structure (Neubert 2011). Size of liposomes plays an important role in the reported penetration of these vesicles into the different skin layers. Verma et al. reported delivery of hydrophilic and lipophilic molecules across the skin and concluded that for both type of molecules, larger nano-carriers (>600 nm) do not penetrate into the deeper layers of the skin and remain localized in the SC, whereas smaller particles (<100 nm) will penetrate into deep layers of the epidermis and in the dermis. However, it is important to note that each formulation needs to be optimized for particle size (Verma et al. 2003). Liposomes have been reported to penetrate and accumulate in the upper layers of the SC but not penetrate deep into the epidermal layers (El Maghraby et al. 2006; Schaller and Korting 1996). For some of these studies, it is not clear if liposomes actually penetrate the skin or just deliver the drug by fusing with the superficial layers of the skin. Aphios® has developed a small, uniform less than 1 nm in diameter liposomal nano-carrier (Nanosomes) called as Dermos™ for the treatment of AIDS-associated Kaposi's sarcoma and other skin cancers (Table 1.1). Aphios® reports that formulation of antineoplastic agent in Nanosomes will help in reducing blood toxicity levels and improve patient's quality of life. The company is seeking for partners to further develop Dermos™ topical formulation (Aphios 2013), while in the cosmetic industry, L'Oreal, Paris, has formulated nanosomal Revitalift® cream and lotions containing Pro-retinol A® for antiaging (Paris 2013). Several authors reported that increasing the elasticity of liposomes could result in enhanced drug penetration across the skin. Also, to overcome other limitations of conventional liposomes such as stability and rigidity, leakage-resistant flexible liposomes were invented. Ethosomes are a type of flexible liposomes mainly composed of phospholipids and relatively high concentration of ethanol and water which are typically in the size range of 10 nm to microns (Elsayed et al. 2006). Ethanol concentration plays an important role in particle size of these ethosomes. Studies have shown that increasing the concentration of ethanol will decrease the particle size of ethosomes, whereas increasing the phospholipid content would increase the size of ethosomes. Presence of ethanol in vesicles produces fluidizing effect which interferes with the lipidic matrix and enhances their fluidity. Several studies have been conducted to show penetration of ethosomes into deeper layers of the skin as compared to liposomes which were present in the superficial layers of the SC (Bhalaria et al. 2009; Celia et al. 2012; Godin and Touitou 2003). Transfersomes are another type of flexible liposomes which are also termed as deformable or ultradeformable liposomes. Along with phospholipids, they also contain an edge activator such as sodium cholate (Celia et al. 2012). They are highly elastic in nature and are known to squeeze through a pore ten times smaller than their size (Elsayed et al. 2006; Gupta et al. 2005). The mechanism of transfersomes' transport into skin is hydration driven, as they travel toward the higher hydration gradient presented by the blood circulation under the epidermis (Cevc and Gebauer 2003). Guo et al. studied the influence of flexible lecithin vesicles on delivery of cyclosporine A (nonpolar oligopeptide made up of 11 amino acids) as compared to conventional vesicles. Sodium cholate was incorporated

Table 1.1 Role of nanotechnology in skin delivery of drugs

Type of carrier	Description	Active	Indication	Marketed by
Nanosomes	Dermos™ nanosomal formulation of and antineoplastic agent with diameter less than 1 nm	Paclitaxel	Treatment of AIDS-associated Kaposi's sarcoma	Aphios®
	Revitalift	Pro-retinol A	Antiaging	L'Oreal
Micellar nanoparticle	Estrasorb topical emulsion with systemic delivery of 0.05 mg/day	Estradiol hemihydrate	Prevention of hot flushes and treatment of vasomotor symptoms associated with menopause	Graceway Pharmaceuticals, LLC
Nanocolloid	Platinum silver nanocolloid cream	Platinum powder, silver, hydrating botanicals, coenzyme Q10	Antiaging	DHC USA, Inc.
Nanocrystal	SILCRYST™ antimicrobial dressing coupled with nanocrystalline silver	Silver	Helps maintain moist wound environment which facilitates faster healing in case of infections	NUCRYST Pharmaceutical Co. (SILCRYST) and Smith and Nephew (ACTICOAT™)
	Cellular serum platinum rare	Hesperidin, colloidal platinum, resveratrol	Antiaging	La Prairie, Switzerland
Dendrimer	VivaGel® Dedrimer, i.e., branched nanoparticles technology. Completed phase 2 trials successfully	SPL7013	Treatment of bacterial vaginosis	Starpharma Holdings
Nanoemulsion	NanoStat™ topical formulation technique uses high energy oil-in-water emulsion to specifically target microbes	NB-001	Treatment of cold sores associated with herpes labialis	Phase 3
Nanolipid	Cutanova nanorepair cream and lotion	Coenzyme Q10, polypeptide, hibiscus extract, ginger extract, ketosugar	Antiaging	Dr. Rimpler
	IOPE Supervital Cream, serum eye cream	Coenzyme Q10, ω-3, and ω-6 unsaturated fatty acid	Moisturizer	AmorePacific

for flexibility, while the rigid vesicles were made without cholate. Though the size range of all vesicles was in the nanorange (61–74 nm average diameter), the flexible vesicles had smaller polydispersity due to the presence of sodium cholate. Flexible vesicles delivered 1.16 ± 0.26 μg through mice skin at the end of 8 h, while rigid vesicles showed no delivery (Guo et al. 2000). El Maghraby et al. reported the use of highly flexible or ultradeformable liposomes. Skin delivery of 5-fluorouracil was enhanced using these vesicles as compared to the traditional liposomes (El Maghraby et al. 2001). Poorly water-soluble drug temoporfin which is a second-generation candidate used for photodynamic therapy was considered for targeting cutaneous malignant as well as nonmalignant diseases by encapsulation in invasomes. Terpenes were found to play an important role in properties of these vesicles thus differentiating them from other types of liposomes (Dragicevic-Curic et al. 2008, 2009). As reported in the literature, terpenes are known to increase

drug partitioning into the skin. The ultradeformable property of invasomes is due to the penetration-enhancing effect of phospholipids, ethanol, and mixture of different terpenes added to them (Verma et al. 2003; Williams and Barry 2004). As discussed, liposomes do not permeate deeper into the skin layers and are known to be retained in the upper layers of the SC, whereas deformable liposomes like transfersomes and ethosomes are known to squeeze into the SC and enter the viable epidermis and dermis region (Gupta et al. 2012).

1.2.2 Solid Lipid Nanoparticles

A more recent nano-carrier is the solid lipid nanoparticle (SLN) (Muller et al. 2000). As the name suggests, SLN is composed of solid lipids dispersed in liquid media and stabilized with surfactants. Typically, lipids range from 0.1 to 30 % w/w, and surfactants are usually anywhere between 0.5 and 5 % w/w. The particle size of SLN is reported to be ranging between 40 and 1000 nm. Loading capacity of SLN is up to 10 % of the lipid amount to ensure stability of the formulation. SLNs are composed of solid lipids assembled in a high-energy crystallized form (α and β′) which eventually convert into low-energy form (β′) resulting in increased orderliness of the system; the subsequent decrease in imperfection leads to drug loss or leakage from the carrier. This limitation gave rise to second-generation lipid carriers – the nanostructured lipid carrier or NLC (Pardeike et al. 2009). NLCs belong to the second-generation lipid nanoparticles technology and are composed of blends of liquid lipids and solid lipids. Drug is incorporated in a less ordered matrix of lipids in NLCs, and this makes the carriers more stable to leakage, biodegradable in nature, and less toxic/nontoxic. NLCs were developed with an aim of overcoming the drawbacks associated with SLNs (Pople and Singh 2011). Das et al. performed a study to compare the efficacy and stability of SLN vs. NLC carriers in delivering clotrimazole (antifungal drug with high lipophilicity) topically. Stability studies were performed at 25 °C for a period of 3 months; NLCs demonstrated greater stabilizing effect and the drug release profile remained the same at the end of 3 months, whereas SLNs exhibited a significant change in release profile during the same period (Das et al. 2012). Gokce et al. reported better skin penetration efficiency of NLCs; they were deposited at higher concentration in the dermis due to small particle size [NLC (90–148 nm) vs. SLN (161–718 nm)] which aided in particle penetration and cell endocytosis (Gokce et al. 2012). However, conflicting reports have also been reported in literature, and superiority of NLCs over SLNs has been questioned in some cases (Lombardi Borgia et al. 2005; Souza et al. 2011). The mechanism by which SLN and NLC enhance delivery is partly by producing occlusive effect on the skin due to the adhesiveness of these nano-carriers on the skin. They are also known to penetrate into the skin via follicular route. Most of the products available on the market containing lipid nano-carriers are for cosmetic applications. Lipid particles, i.e., more specifically SLNs and NLCs, offer advantages like stabilization of the active agents, achieve desired degree of occlusion and subsequent hydration of skin, improved shelf life of the final product, and higher bioavailability and site-specific action (Muller et al. 2007). NLCs are carriers for many cosmetic products; a few examples are Nanorepair Q10 cream and serum (Dr. Rimpler GmbH, Germany), NLC deep effect series (Beate Johnen, Germany), and IOPE (AmorePacific Corporation, South Korea), among many others. Coenzyme Q10 is an antioxidant present more abundantly in the epidermis (10 times higher than in the dermis), and decrease in its skin levels is associated with skin aging. The formulation of coenzyme Q10 for an antiaging therapy is a popular approach in cosmetic industry; the first NLC product for the same was launched in 2005 by Dr. Rimpler GmbH, Germany, known as Cutanova Cream Nanorepair Q10 (Muller et al. 2007). Pardeike et al. evaluated Cutanova Q10 cream vs. oil-in-water conventional cream based on parameters relevant to cosmetic appeal of a product including consistency, spreadability, and hydration as well as product properties like particle size and rheology. The authors concluded that the NLC formulation was stable and had no irritant

action. Volunteers rated the NLC formulation better in terms of feel on the skin and hydration, suggesting that NLCs were favorable vehicles for cosmetic preparations (Pardeike et al. 2010). Nanoemulsions are another type of nano-sized carriers which form oil and water emulsions; they help in achieving stabilization, better penetration, and targeted delivery of drug in the skin. NanoBio Corp., USA, has developed a nanoemulsion topical formulation containing antimicrobial agent. The nanoemulsions are nanometer-sized oil-in-water emulsion containing positively charged droplets having average particle size of 180 nm (Table 1.1) (Corporation 2013).

1.2.3 Polymeric Nanoparticles

Polymeric nanoparticles are made up of biodegradable, nontoxic, and bioadhesive polymers such as chitosan, polycaprolactone, polylactic-glycolic acid (PLGA), and polystyrene (Senyigit et al. 2010; Shah et al. 2012; Wu et al. 2009b). The benefits offered by polymeric nanoparticles include, but are not limited to, increased localized targeting and reduced systemic side effects, lower irritation, better control on release of therapeutic agent by altering the polymer properties, and prevention of breakdown of susceptible drugs by encapsulating them (Alvarez-Roman et al. 2004a; Haag and Kratz 2006; Kuchler et al. 2009b; Wu et al. 2009b). Polymeric nanoparticles, due to their rigid nature, cannot penetrate the SC intact. Due to bioadhesiveness, they stick to the furrows on the skin and release their drug content into the deeper layers of the skin (Desai et al. 2010). However, they can accumulate in the hair follicles and travel into the deeper regions in a size-dependent manner. Alvarez-Roman et al. studied distribution of polymeric nano-carriers (diameters 20 and 200 nm) across porcine skin. CLSM images showed that the nanoparticles accumulated in the hair follicles in a time-dependent manner. This localization was also dependent on the particle size where higher accumulation was demonstrated by the smaller particles (Alvarez-Roman et al. 2004b). Imaging studies have confirmed that polymeric nano-carriers penetrate into the skin via follicular route and do not permeate into the SC (Campbell et al. 2012). Kuchler et al. compared dendritic core-multishell nanotransporters (CMS) with SLN for delivery of lipophilic dye nile red and hydrophilic dye rhodamine B. The polymeric CMS could entrap the hydrophilic dye more efficiently due to the presence of polyethylene glycol, whereas incorporation in SLN was low owing to high lipid content of the carrier. Furthermore, CMS nanotransporters not only led to a 13-fold increase in penetration of lipophilic dye (nile red) in the viable epidermis but also an 8-fold increase in the SC. The enhancement in penetration was higher than that achieved for SLN. Moreover, the hydrophilic dye did not show accumulation in the SC for both the carriers due to lack of retention in this skin layer. CMS nanotransporters achieved higher uptake of both the dyes, nile red and rhodamine B, into the viable epidermis over SLN as they had a favorable particle surface-to-volume ratio. However, the authors after comparing CMS nanotransporters, SLN, and lipid nanocapsules (LNC) concluded that rather than particle size, other characteristics of vesicles may be a decisive factor in dermal uptake, and further studies are needed to confirm the same. (Kuchler et al. 2009b). Batheja et al. formulated the model compound diclofenac sodium and the lipophilic dye nile red in tyrosine-derived nanospheres dispersed in a hydrophilic gel with a penetration enhancer Azone (1-dodecylazacycloheptan-2-one, laurocapram) for achieving higher drug delivery. Results showed that gel formulation achieved significantly higher permeation (1.4–1.8-fold) as compared to the aqueous formulation; this was attributed to the higher viscosity of the gel formulation which allowed better contact with the skin (Batheja et al. 2011). However, a balance needs to be established in some cases between the hydrophilicity and hydrophobicity of the carrier for optimum delivery of a drug into the skin as reported by Wu et al. (2009a, b). Nanoparticle dispersions of nile red were prepared in a polymeric mixture of polystyrene (PS) and poly-(2-hydroxyethyl methacrylate) (HEMA) at varying proportions, and its penetration into skin was determined by tape stripping. The results

showed that increasing percentage of HEMA, i.e., the hydrophilic polymer, led to the decrease in skin penetration of nile red as the hydrophobicity of the carrier was compromised. Tape-stripping data showed PS, nanoparticle dispersion showed highest amount of nile red in the SC, and this was statistically significant from other groups, i.e., PS+ 5 % HEMA, PS+ 10 % HEMA, and PS+ 20 % HEMA. Confocal microscopy performed to trace the path of particles also reaffirmed that the composition of the nanoparticle dispersion determined their successful delivery into the skin (Wu et al. 2009a, b). Polymersomes are amphiphilic vesicles consisting of hydrophilic-hydrophobic block copolymers assembled in bilayers; they have the advantage of being more robust and display greater stability than their lipid counterpart liposomes (Kim et al. 2011). Rastogi et al. reported confocal images showing delivery of nile red-loaded flexible polymersomes across SC and into deeper layers of the viable epidermis. The first sites of uptake are surface ridges or furrows for colloidal carriers, whereas the second sites of penetration are the hair follicles as confirmed by images from CLSM at different time points. In addition to the hair follicles which generally act as penetration routes for polymeric nanoparticles, increased fluorescence in the intercellular region was also seen and attributed to the deformable nature of the polymersomes; these flexible vesicles are also capable of squeezing their way through the narrow hydrophilic channels or creating pores in the epidermis for short durations (Rastogi et al. 2009). Surface modification of polymeric nanoparticles is another approach to enhance penetration into the skin, e.g., "NanoEase" technology aims at improving delivery of nanocarriers deeper into the skin by surface modification of poly(lactic-co-glycolic acid)-chitosan nanoparticles with oleic acid. Shah et al. delivered significantly higher amounts of a combination of anti-inflammatory drugs, ketoprofen and spantide II, into the epidermis and dermis using this technology. In vivo study with the aforementioned combination performed in allergic contact dermatitis mice model also showed reduced inflammation (Shah et al. 2012). Starpharma Holdings (Australia) VivaGel®, a branched polymeric nano-carrier [poly(amido amine) dendrimers], has successfully completed phase 2 clinical trials for the treatment of bacterial vaginosis. VivaGel® is also used to coat the condoms for the treatment of HIV and sexually transmitted infections (STI) (Holdings 2013).

1.2.4 Nanocrystals

About 40 % of the new drugs discovered are poorly soluble in water. Reduction in particle size of a drug molecule can increase the surface area thereby increasing the dissolution rate and ultimately leading to increase in saturation solubility. Nanocrystals were first used for oral drug delivery in the early 1990s (Muller and Keck 2012). Nanocrystals exhibited several properties like increased penetration into a membrane, increased adhesion, and enhanced permeation through the gastrointestinal wall; these properties were then used to apply nanocrystals for topical application (Muller et al. 2011). The nanocrystal technique produces particles below the micron size with either amorphous or crystalline structure (Chen et al. 2011). They are 100 % drug particles stabilized in aqueous medium with the help of surfactants (Junghanns and Muller 2008; Muller et al. 2011). Drug molecules which have been formulated as nanocrystals to improve topical application include diclofenac sodium, hesperetin, lutein, and resveratrol, hesperidin, and apigenin (Al Shaal et al. 2011; Kobierski et al. 2011; Mishra et al. 2009; Mitri et al. 2011; Müller et al. 2011; Shegokar and Muller 2010). The mechanism by which nanocrystals penetrate the skin is explained as based on the size and solubility parameter. Nanocrystals are incorporated in the aqueous phase of the dermal formulation, i.e., cream, gel, or lotion; high dissolution of drug in the form of nanocrystals causes increased saturation solubility which further enhances the concentration gradient between the formulation and skin leading ultimately to better penetration of the drug. As the drug is delivered through the skin, nanocrystals also act as a reservoir by releasing more drug into the water phase of a

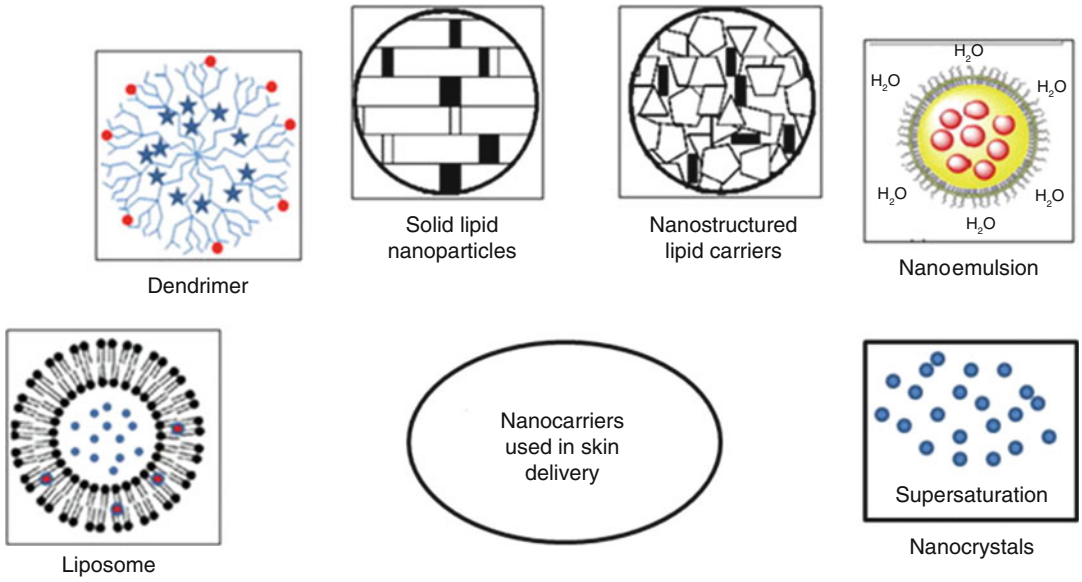

Fig. 1.2 Different types of nano-carriers used for delivery in the skin

cream/gel/lotion (Shegokar and Muller 2010). NUCRYST Pharmaceutical Corp., Canada, has patented SILCRYST™ products which are nanocrystals of silver that dissolve faster than microcrystalline silver; thus, rapidly available silver exerts its antimicrobial activity. SILCRYST™ is coated on a medical dressing. Combination of these two technologies, i.e., SILCRYST™ on medical dressing, is called as ACTICOAT™ dressings for topical application in treating infections and inflammatory conditions. ACTICOAT™ dressings provide fast antimicrobial action and fewer dressing change providing more comfort to the patients (Corp 2013) (Fig. 1.2).

1.3 Success in Pharmaceutical Industry

Nanotechnology has been studied and is of great interest to different industries including electronic, automotive, textile, food, cosmetics, and pharmaceutical. Extensive research has led to introduction of nanotechnology in our daily lives, and efforts to incorporate more such approaches continue. Topical and transdermal routes of delivery using various nano-carriers have been discussed in this chapter, and it is evident that nanotechnology offers a huge potential to successfully transport of drugs into and through the skin. However, the studies conducted in the laboratories have not yet translated into commercial success for pharmaceutical industry as well as for the cosmetic industry. The first cosmetic formulation containing nano-carriers was the liposome-containing antiaging gel Capture™ launched by Christian Dior, France, in 1986. Lipid-based nano-carriers are one of the most researched vehicles as mentioned earlier in this chapter, and the first cosmetic product containing NLC was marketed in 2005 by Dr. Rimpler GmbH, Germany. Since then at least 30 cosmetic products using lipid-based nano-carriers have been marketed worldwide. Table 1.1 provides an insight to the products which have been marketed or have a strong potential for the same.

1.4 Limitations

The skin is a robust barrier, and its significance in protecting the human body from exogenous agents is important when designing any topical/transdermal drug delivery system (Desai et al. 2010). The approval and marketing of nanotherapeutics

require them to meet safety guidelines established by government agencies. Health and environmental concerns need to be addressed before the products become available to the patients. The choice of polymers/excipients determines the toxicity of nano-carriers in vivo, as the trend has shifted to incorporation of biodegradable agents which ensure the removal from body after a stipulated period of time. The FDA has draft a guidance on safety assessment of products in cosmetics; however, no premarketing approval is required. The European Commission has introduced guidelines in July 2012 which include informing the commission 6 months prior to marketing of a product incorporating nanomaterials and risk assessment of the same for safety purposes. Another limitation and an area which needs extensive standardization is the manufacturing of these nano-carriers (Gupta et al. 2012). The manufacturing procedures need to be studied on a larger scale since taking the technology from lab to industrial level may present its own set of challenges. Size range and increased surface area are the main advantages of nanoparticulate systems; however, these same characteristics are responsible for safety concerns associated with them. Human exposure to nanoparticles can be accidental (e.g., environment and people working in manufacturing units) or desired (use of cosmetics and medicines). Moreover, the skin is an easily accessible organ which comes in contact with exogenous matter. In this chapter, we have talked about different types of nano-carriers and the general trend toward use of materials which are biocompatible as well as biodegradable with the human body. Lipid nanoparticles are formulated from phospholipids and lipid components similar to that present in human skin. Rigid polymeric nano-carriers do not traverse the skin layers; they act by releasing the drug into the skin, while the vesicles remain occluded to the skin surface or get deposited in the hair follicles depending on the size range as mentioned (Baroli 2010). However, as most of the polymers used are biodegradable and nontoxic to human skin, any concern related to material of nano-carrier system is minimized. Formulation-specific analysis is needed for each nanoparticle system as presence of other components like ethanol (ethosomes), edge activators (transfersomes), and penetration enhancers like Azone and terpenes (polymeric nanoparticles and invasomes) can influence the irritancy and toxicity. Risk assessment of nanoparticles applied to skin is an important area especially when dealing with conditions that compromise skin integrity, i.e., inflammation, diseased skin, or damaged skin. Literature on this topic is restricted due to lack of established methods and required equipment to detect accurately the toxicity of nano-carriers in the skin (Stern and McNeil 2008). Parameters like particle shape, size of nano-carriers, drug-loading capacity, and drug release should be reproducible (Prow et al. 2011). As discussed earlier in the chapter, different types of nano-carriers can be fabricated; however, this comes with its set of challenges as each group has to be assessed according to a specific criteria, keeping in mind the properties and mechanism of action of the system.

Conclusion

Several nano-carriers such as liposomes, transfersomes, ethosomes, SLN, NLC, and other polymeric nanoparticles have been developed and investigated for delivery of drugs and cosmeceuticals. Nanotechnology-based drug delivery helps protect the active drug from degradation thereby increasing formulation stability, releases the drug in a controlled manner, reduces skin irritancy, and increases drug loading. Nano-carriers accumulate in the hair follicle leading to faster and more efficient delivery of drug or cosmeceutical, especially for localized delivery such as for acne. Smaller nano-carriers may also penetrate the superficial layers of the stratum corneum and deliver the incorporated drug into the viable epidermis and dermis. Specially tailored nano-carriers can be also used for transdermal drug delivery. The cosmetic industry has successfully launched products which are available for skin delivery. However, the pharmaceutical industry has yet to see a nanotechnology product in the market, and this may be due to safety con-

cerns associated with nanotechnology and limited risk assessment studies of nanoparticles on skin. More research needs to be done in this field to make skin delivery via nano-carriers a consumer-friendly approach.

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Liposomes as Drug Delivery Systems in Dermal and Transdermal Drug Delivery

2

Slavica Siler-Marinkovic

Contents

2.1 Historical Perspectives

The term liposome is derived from two Greek words: "lipos" (fat), and "soma" (body). The term liposome was originally used in the literature before 1950 for colloidal aggregates of triglycerides which are very different from the lipid vesicles described in this review (Lasic 1996).

Liposomes were the first vesicular carrier studied for the delivery of drugs into the skin. The liposome story began with a paper in 1964, published in the *Journal of Molecular Biology*, in which Bangham and Horne from the Babraham Institute in Cambridge showed electron microscopic images of multilamellar phospholipid vesicles (Bangham and Horne 1964). The usage of the term liposomes for the description of lipid vesicles was proposed in 1968 (Sessa and Weissmann 1968; Kinsky et al. 1968). As lipid vesicles are often single-bilayer hollow spheres, which have nothing in common with "fat bodies," the use of the term liposome is in principle not

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appropriate (Lasic and Barenholz 1996a, b). Some authors use the term vesicle exclusively for a closed single or bilayer aggregate of amphiphiles (Kaler et al. 1992; Laughlin 1997).

Initially, liposomes were attractive as model systems for biological membranes, because of similarities in lipid composition and structure. Their usefulness as drug carriers was discovered by Sessa and Weissman (1970, 1997), who reported the encapsulation of lysozyme in multilamellar vesicles (MLV). Liposomes have been investigated extensively as a drug carrier system by various routes of administration and are accepted as potential carriers for a variety of drugs that include low molecular weight compounds, therapeutic proteins, diagnostic agents, and cosmetic actives (Egbaria and Weiner 1990; Du Plessis et al. 1994; Cevc 1996; Cevc and Vierl 2010; Kirjavainen et al. 1999; Walde and Ichikawa 2001; Blume et al. 2003; Choi and Maibach 2005; El Maghraby et al. 2008; Shailesh et al. 2009).

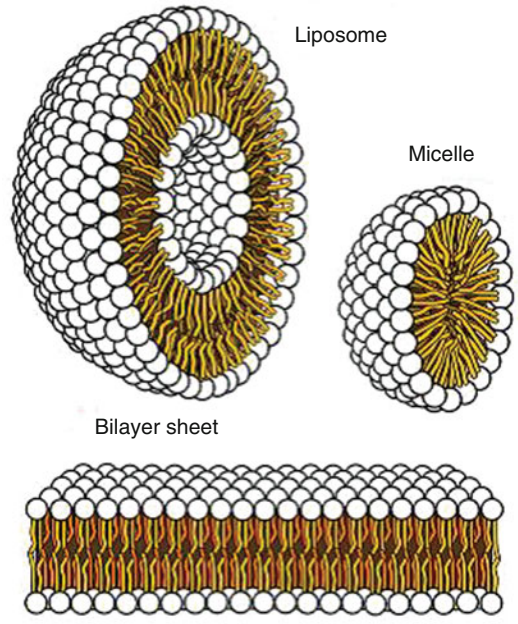

Fig. 2.1 Liposome structure (http://en.wikipedia.org/wiki/File:Liposome_cross_section.png)

2.2 Mechanism of Vesicle Formation

Liposomes are spherical vesicles, 0.05–5.0 μm in diameter, whose membranes consist of one (unilamellar) or more (oligolamellar, multilamellar) bilayers of phospholipids. The amphipathic nature of phospholipids and their analogues render them the ability to form closed concentric bilayers in the presence of water. When lipids are exposed to an aqueous environment, interactions between themselves (hydrophilic interactions between polar headgroups and Van der Waals interactions between hydrocarbon chains) and with water (hydrophilic interactions, hydrophobic effect) lead to spontaneous formation of closed bilayers. Spontaneous aggregation is not only determined by the hydrophobic contribution but is also related to the molecular parameters of the amphiphile (Lautenschläger 2006) (Fig. 2.1).

Liposomes may be characterized for their size, lamellarity, capture volume capacity, method of preparation, chemical integrity of the lipids, and application. Classification of liposomes based on structural parameters is represented in Fig. 2.2.

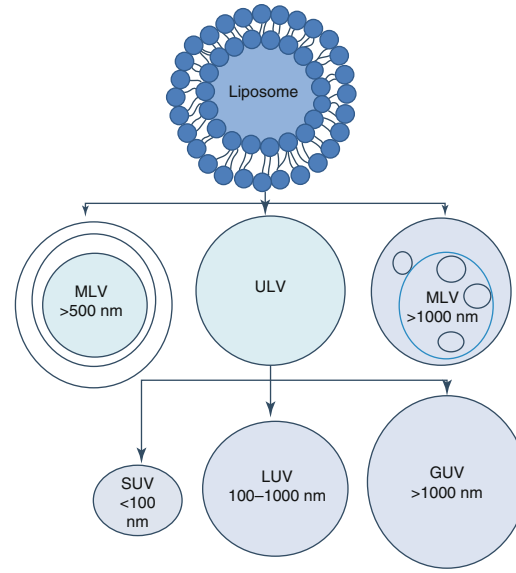

Fig. 2.2 Types of liposomes based on structure (Lautenschläger 2006)

2.3 Material for Vesicle Formation

Liposomes can be composed of naturally derived phospholipids with mixed lipids, hydrogenated

phospholipids, or of pure surfactants. Major structural components of conventional liposomes are phospholipids and cholesterol.

2.3.1 Phospholipids

Phospholipids, the major structural component of biological membranes, are amphiphilic with hydrophobic hydrocarbon tail and hydrophilic polar head (Fig. 2.3). The tails are repelled by water and line up to form a surface away from the water. The hydrocarbon tails of one layer face the hydrocarbon tails of the other layer, and the combined structures form a bilayer.

The glycerophospholipids are the most common used component of liposome formulations, derived from phosphatidic acid. Examples of natural phospholipids are phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), and phosphatidylglycerol (PG).

The lipid composition determines the physical characteristics of the liposomes and the

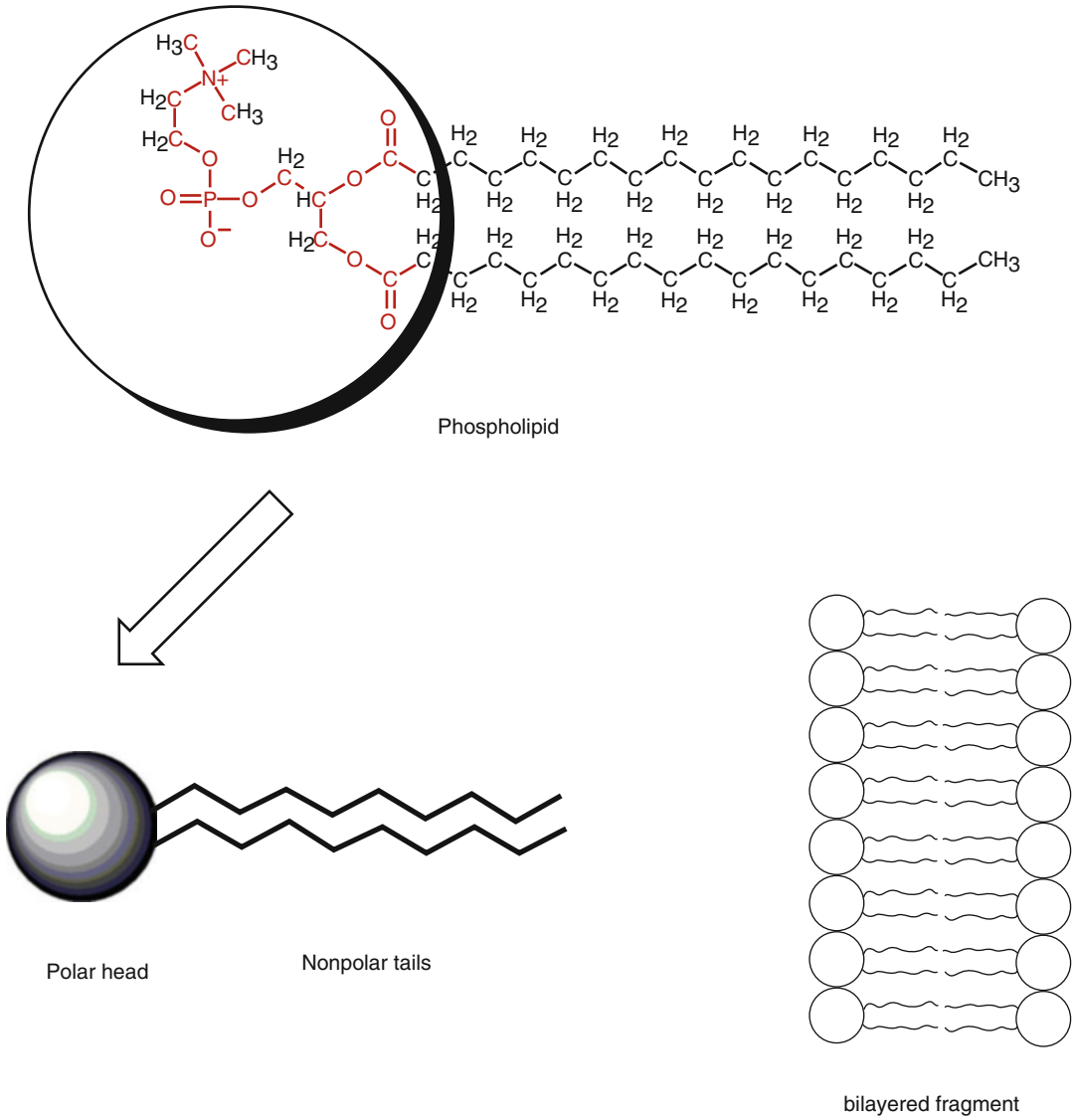

Fig. 2.3 Phospholipid structure

interaction of these carrier systems with the skin (Choi and Maibach 2005).

Natural phospholipids are obtained either from soy beans or eggs, which differ in composition of fatty acids and properties. Egg phospholipids have a higher content of saturated fatty acids (40 % of 16:0 and 18:0) in comparison to soy bean (80 % of 18:1 and 18:2). Soy phosphatidylcholine has a high content of unsaturated linoleic acid and a very low phase transition temperature of below 0 °C. This may be the reason behind its ability to fluidize the lipid bilayers of the horny layer in the skin. Because of its penetration capability, soy phosphatidylcholine delivers linoleic acid very effectively into the skin. By adhering very strongly to skin surface containing keratin, phosphatidylcholine shows moisturizing and softening effects. Liposomes composed of unsaturated fatty acids transported active agents better into the horny layer than liposomes composed of saturated fatty acids. The conditioning effect causes the horny layer to become a depot for these agents, and a more continuous permeation takes place outside the horny layer depot into the living part of the skin, over a longer period of time. This property makes this kind of liposomes very attractive for the application of substances influencing the regenerating ability of the living epidermis. In contrast, this kind of liposomes does not strengthen the natural barrier function of the skin with an exception of its indirect effect of supporting the formation of ceramide I. The liposomes based on natural phospholipids are not very stable, but the cost of purified lipids is very high.

The most common phospholipid used for the production of liposomes is PC. Phosphatidylcholine has a cylindrical molecule not soluble in water, which organizes itself into flat lamellae, i.e., bilayered structures, in order to minimize the unfavorable contact between the bulk aqueous phase and the long hydrocarbon fatty chains, which can further form liposomes. In contrast, other amphipathic molecules, such as detergents, have conical molecules and form micellar structures in water. Phosphatidylcholine membranes, like other lipid membranes, can exist in different phases, such as fluid liquid state and solid gel state, depending on the temperature. At phase transition temperature (Tm), lipid membranes pass from a tightly ordered gel state to a liquid crystal phase, where the freedom of movement of individual molecules is higher. Lipids with higher phase transition temperature have more stable molecules; however, they are at skin temperature in the gel state.

Instead of unsaturated PC, a fully hydrogenated PC should be selected for products with a longer shelf-life. Hydrogenated PC contains mainly stearic und palmitic acids and semisynthetic compounds, like dipalmitoylphosphatidylcholine (DPPC) and distearoylphosphatidylcholine (DSPC). Hydrogenated PC has high Tm in aqueous system of 50–60 °C, and it is insoluble in triglycerides, alcohols, and water. Such liposome preparations have better storage stability.

Recently, liposomes have been prepared using synthetic phospholipids. Commonly used synthetic phospholipids are dioleoylphosphatidylcholine (DOPC), distearoylphosphatidylcholine (DSPC), dipalmitoylphosphatidylcholine (DPPC), dioleoylphosphatidylethanolamine (DOPE), and distearoylphosphatidylethanolamine (DSPE) (Lautenschläger 2006; Kulkarni 2005; Braun-Falco et al. 1998).

2.3.2 PEGylated Lipids

PEGylated lipids have a structure similar to phospholipids, but instead of the first acyl hydrocarbon chain, they have a polyethylene glycol (PEG) chain (Fig. 2.4). Glycerol provides the backbone, and the two hydrocarbon chains R1 and R2 can vary in length. The size of PEG chain can be from 8 to 45 subunits (*n*). The size of the PEG head relative to the length of the hydrophilic chain is the fundamental property that allows liposome formation (Lasic 1998).

This group of novel lipids has properties that allow formation of thermodynamically stable liposomes. PEGylated lipids prolong the plasma half-life of the encapsulated drugs. Coating of the liposomal surface with PEG would suppress the uptake of the drug by the reticuloendothelial system. PEG-12 glyceryl dioleate, PEG-12 glyceryl

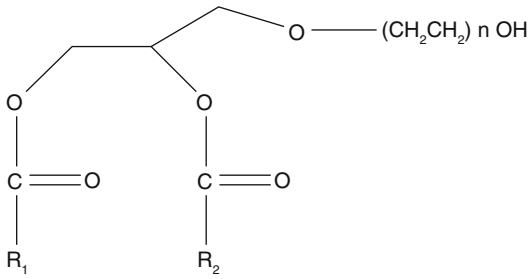

Fig. 2.4 Structure of PEGylated lipid

dimyristate, PEG-23 glyceryl palmitate, PEG-12 glyceryl distearate, and PEG-23 glyceryl distearate are suitable constituents of novel liposomes for cosmetic purposes (El Maghraby et al. 2004).

2.3.3 Polymeric Material

Synthetic phospholipids with diactylenic group in the hydrocarbon chain polymerize when exposed to UV light leading to the formation of polymerized liposomes. Polymerizable lipids contain conjugated diene or methacrylate (Regen et al. 1981).

2.3.4 Cholesterol

Cholesterol cannot form bilayer structures alone. However, it can be incorporated into phospholipid membranes in a very high concentration up to 1:1 or even 2:1 molar ratio (cholesterol to phosphatidylcholine). Cholesterol is included in liposomes for decreasing the fluidity of the bilayer, reducing the permeability of the membrane to water-soluble molecules, and stabilizing the membrane in the presence of biological fluids such as plasma (Praveen et al. 2009).

2.3.5 Other Substances

Varieties of other lipids or surfactants are used to form liposomes. Many single-chain surfactants or nonionic lipids can form liposomes, when mixed with cholesterol. A variety of polyglycerol and polyethoxylated mono and dialkyl amphiphiles are used mainly in cosmetic preparations. Single- and double-chain lipids having fluorocarbon chains can form very stable liposomes. Stearylamine and diacetyl phosphate are incorporated into liposomes to impart either a negative or a positive surface charge to these structures (Praveen et al. 2009).

2.4 Preparation of Liposomes

There are three groups of methods employed to produce liposomes (Fig. 2.5)

- Mechanical methods
- Methods based on replacement of organic solvent(s) by aqueous media
- Methods based on detergent removal

The choice of preparation method depends on the following parameters:

- The characteristics of major structural components of liposomes
- The characteristics, concentration, and toxicity of the material to be entrapped
- The nature of the medium in which the lipid vesicles are dispersed
- Additional processes involved during application/delivery of the vesicles
- Optimum size, polydispersity, and shelf-life of the vesicles for the intended application
- Reproducibility of the method and possibility of large-scale production

2.4.1 Mechanical Methods

The general steps of the production of liposomes are preparation of the lipids for hydration, hydration with agitation, and sizing to a homogeneous distribution of vesicles.

Thin film was the first method for liposome preparation. Lipids are dissolved in organic solvents (chloroform or mixtures with methanol), and the solvent is removed under a high vacuum with a rotor evaporator, forming a thin film on the walls of round-bottomed flask. The aqueous

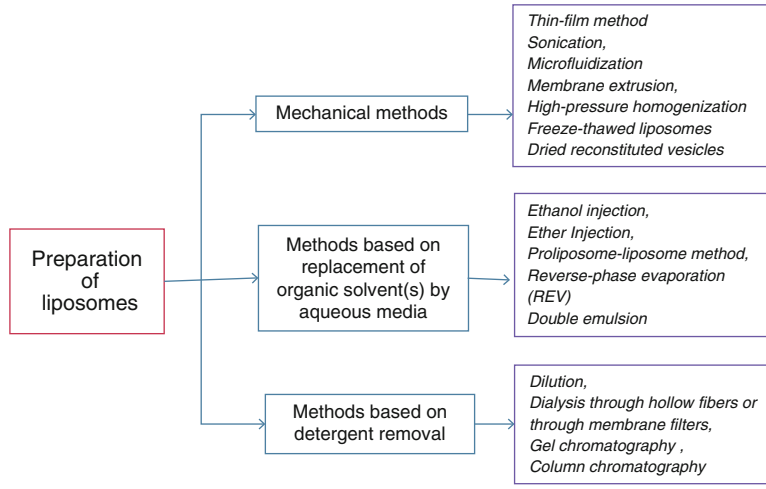

Fig. 2.5 Methods of preparation of liposomes

phase is prewarmed at temperatures above the Tm of the lipids used. After the addition of the aqueous phase, the thin film is detached from the flask walls by agitation and a highly heterogeneous population of MLVs is produced. Vesicle size is influenced by the lipid charge, nature of the aqueous phase, and power input of agitation. Since then, many different variations of this method have been developed. This method is easy to perform, and high encapsulation rates of lipid as well as aqueous soluble substances can be achieved because high lipid concentrations can be used. However, this method is not suitable for industrial production. Furthermore, the process becomes more time-consuming and cost intensive because additional processing is recommended for a defined liposome suspension (Braun-Falco et al. 1998).

The first published *homogenization technique* was sonication. A very high-energy input based on cavitation is applied to the liposomal dispersion either directly with a tip or indirectly in a bath sonicator. The position of the probe plays an important role on the ability to minimize vesicle size. Other methods are homogenization techniques, either by shear or pressure forces, such as microfluidization, high-pressure homogenization, and shear force-induced homogenization techniques. The most defined method for downsizing is the extrusion technique whereby liposomes are forced through filters with well-defined pores (Riaz 1996).

2.4.2 Methods Based on Replacement of Organic Solvent(s) by Aqueous Media

The liposome preparation methods described in this section have in common that organic solvents, either water miscible or immiscible, are replaced by an aqueous solution. This replacement is either performed by injection of the lipid carrying organic solution into the aqueous phase—the injection methods—or by stepwise addition of aqueous phase to the organic phase, in particular ethanol—the proliposome liposome method (Batzri and Korn 1973).

Ethanol injection method is a good alternative for the preparation of SUVs without sonication, based on injection of ethanolic solution of lipids into rapidly stirred aqueous buffer phase. By the immediate dilution of the ethanol in the aqueous phase, the lipid molecules precipitate and form bilayer planar fragments, which form liposomal systems, thereby encapsulating aqueous phase. The lipid concentration in ethanol is the only liposome formation influencing parameter. The method is very easy, but has an extra step to remove ethanol, and the concentration of vesicles produced and encapsulation of hydrophilic drugs is rather low (Kremer et al. 1977).

Ether injection method is a very similar method, and the only difference is that the lipid is injected slowly in the aqueous solution that is warm.

The proliposome-liposome method is based on the conversion of the initial proliposome preparation into a liposome dispersion by dilution with an aqueous phase (Jung et al. 2002). This method is suitable for the encapsulation of a wide range of drugs with varying solubility in water and alcohol and has extremely high encapsulation efficiencies.

In addition, the emulsification methods, namely, the *reverse-phase evaporation method (REV)* and the double emulsion technique, are based on the replacement of a water-immiscible solvent by an aqueous phase, thus forming liposomes with high encapsulation rates of hydrophilic as well as lipophilic substances (Szoka and Papahadjopoulos 1978).

2.4.3 Methods Based on Detergent Removal

In this group of methods, the phospholipids are brought into contact with the aqueous phase via detergents, which associate with phospholipid molecules and serve to screen the hydrophobic portion of the phospholipid molecule from water. In contrast to lipids, detergents are highly soluble in both aqueous and organic media. There is an equilibrium between the detergent molecules in the aqueous phase and the lipid environment of the micelles. Common used detergents are bile salts or alkylglycosides. The size, shape, and homogeneity of the resulting vesicles depend on the chemical nature of the lipids and detergent, their concentration, and the method and rate of detergent removal. Although liposomes are produced under mild conditions, this method suffers from low encapsulation efficiency of hydrophilic drugs (Schubert 2003).

Common procedures for detergent removal from the mixed micelles are dilution, gel chromatography (Schurtenberger et al. 1984), and dialysis through hollow fibers (Schurtenberger et al. 1976) or through membrane filters (Goldin 1979). Additionally, detergents can also be removed by adsorption to hydrophobic resins or cyclodextrins (Milsmann et al. 1978).

2.4.4 Large-Scale Industrial Production

In contrast to the many pessimistic forecasts during the 1980s, the reproducible preparation of large volumes of stable liposomes no longer presents a problem. They are prepared from well-characterized raw materials with established safety profiles. Furthermore, in most cases, shelf-life stability problems have been successfully solved as well. A characteristic liposome formulation consists of several different lipids that have to be mixed before hydration; normal practice is to lyophilize the lipid mixture from tert-butanol or to use a spray-dried lipid powder, or a thoroughly dried thin film. What is next performed? Adding organic solvents such as chloroform or methylene chloride to solubilize and mix lipids is not recommended (US regulations allow 50 ppm of chloroform and 500 ppm of methylene chloride in the formulation) (Lasic 1998).

Alternatively, lipid solutions in water-miscible (ethanol, propylene glycol) or water-immiscible (ether, freon) organic solvents can be injected into an aqueous phase followed by organic solvent removal (and possibly recycling) by evaporation, filtration, or dialysis. For cosmetic and some nutritional products, this may not be needed because the remaining solvent (propylene glycol or ethanol) may not be harmful and, additionally, prevents microbial growth. At higher lipid concentrations, all these hydration methods give rise to large multilamellar liposomes, which can be converted into smaller, unilamellar liposomes by extrusion and homogenization techniques (Riaz 1996).

A more suitable method for large-scale production of liposomes is "microfluidization." This process is similar to the "French Press" method. In this method, slurry-like concentrated lipid/water dispersions are introduced into the microfluidizer which then pumps it at a very high pressure (10,000–20,000 psi) through filters of 1–5 μm pore size. The fluid moving at a very high velocity is split into two streams by forcing them through two defined microchannels. The two streams are then made to collide together at right

angles at very high velocity. The fluid collected at the end is re-passed until a homogeneous dispersion is obtained. The microfluidization technique typically produces unilamellar liposomes of 50–500 nm diameter. The size of the liposomes can be roughly controlled by the processing pressure at which a microfluidizer is operated. Unfortunately, certain active ingredients, particularly proteins, may break down under the high processing pressure, which makes the microfluidization technique unsuitable for such actives (Vemuri et al. 1990).

In contrast to the microfluidizer, where the fluid stream is split and mixed by collision in a mixing chamber, homogenizers work on a different principle. In a homogenizer, the fluid beam is pressed with high pressure through an orifice, and this beam collides with a stainless steel wall. The liposome suspension is continuously pumped through the homogenizer system, where high pressures are generated to downsize lipid vesicles (Brandl et al. 1990).

The most prominent downsizing method is the extrusion. In this method, preformed vesicles are forced through defined membranes by a much lower pressure. Extrusion through polycarbonate filters was first published by Olson et al. in 1979. Depending on the apparatus and scale, the diameters of these membranes range from 25 to 142 mm (Mayer et al. 1986). As suggested for all downsizing methods, liposomes should be extruded above the Tm of the lipid composition. The main disadvantage of this method is the long-lasting preparation starting with preformed liposomes, eventually an additional freeze-thaw step, and finally the extrusion. In these procedures, high product losses may be generated, especially if clogging of the extrusion membranes occurs which may cause technical limitations with large-scale production (Table 2.1).

Table 2.1 Methods of liposome preparation and the resulting product

Method	Type of vesicles, size, nm	Encapsulation volume µl/µmol lipid	Encapsulation efficiency % active encapsulated (water soluble)
Mechanical methods			
Vortex or hand shaking of phospholipid dispersions	MLV 100–1000 nm		
French Press or microfluidization	LUV 100–1000 nm	2–7	Up to 50
French Press or microfluidization and harvesting by ultracentrifugation	SUV	0.2–0.5	Up to 15
High-pressure homogenization	Mainly SUV	0.2–0.5	Up to 15
Sonication followed by ultracentrifugation	SUV 20–100 nm	0.2–0.5	Up to 15
Extrusion through polycarbonate filters at low or medium pressure	OLV, LUV		
Dehydration-rehydration : Liposomes are stored freeze dried, rehydrated when required	MLV 100–1000 nm	2.5–6	Up to 60
Methods based on replacement of organic solvent(s) by aqueous media			
Removal of organic solvent(s)	MLV, OLV, SUV		
Hydration of dry lipid film followed by 3–10 freeze thaw (thawing to>Tm) cycles	MLV 100–1000 nm	2.5–6	Up to 60
Ethanol injection followed by extrusion	LUV, 100–1000 nm	2–7	Up to 50
Ether infusion (solvent vaporization)	LUV, OLV, MLV		
Reverse-phase evaporation	GUV, 1000 nm	As high as 720	Up to 60
Methods based on detergent removal			
Gel exclusion chromatography	SUV		
"Slow" dialysis	LUV, OLV, MLV		
Fast dilution	LUV, OLV		

Partly from Lasic and Barenholz (1996a)

2.5 Formulating with Liposomes

In formulating pharmaceutical or cosmetic products with liposomes, procedures and raw materials must be considered carefully to avoid adverse effects on liposome stability. In general, liposomes should be added to a formulation below 40 °C using low shear mixing. The addition of liposomes should also be the last step in the formulation's manufacturing process. Ethyl alcohol concentration should be kept below 5 %, solvents should be kept below 10 %, and high levels of salts (<0.5 %) should be avoided. Surfactants in general should also be avoided, but low levels (up to 1 %) of nonionic surfactants of high HLB values are usually well tolerated. Acceptable preservatives for liposome formulations include phenoxyethanol (and) methylparaben (and) ethylparaben (and) butylparaben (and) propylparaben (and) isobutylparaben. The recommended storage temperature of most liposome formulations is 25 °C (Braun-Falco et al. 1991).

2.6 Types of Liposomes

Specification of liposomes based on the structure of the vesicle and application includes many types of liposomes like conventional liposomes and novel liposomes [elastic liposomes, long-circulating liposomes, coated (lipoprotein-, carbohydrate-, PEG-coated), liposomes or sterically stabilized (Stealth®, manufacturer, country) liposomes, ethosomes, niosomes, Transfersomes® (manufacturer, country), invasomes, proliposomes, proniosomes, pharmacosomes, vesosomes, Marinosomes® (manufacturer, country), Rovisomes® (manufacturer, country), etc. (Table 2.2). Some of these liposomes are used for parenteral drug administration, but for dermal and transdermal application, conventional and flexible liposomes are still used.

Conventional liposomes can be defined as liposomes that are typically composed of only phospholipids (neutral and/or negatively charged) and cholesterol. Most early studies on liposomes as a drug carrier system employed this type of liposomes. They can vary widely in their physicochemical properties, such as size, lipid composition, surface charge and number, and fluidity of the phospholipid bilayers. Important derivatives of conventional liposomes are nonionic surfactant-based liposomes called niosomes (Choi and Maibach 2005).

Flexible (elastic) liposomes are similar to conventional liposomes but with the incorporation of an edge activator in the lipid bilayer structure to provide elasticity. Elastic liposomes are applied nonoccluded to the skin and have been shown to permeate through the SC lipid lamellar regions as a result of the hydration or osmotic force in the skin. They have been investigated as drug carriers for a range of small molecules, peptides, proteins, and vaccines.

Some of flexible liposomes are small, unilamellar vesicles (80–250 nm) prepared of soy PC (>80 %) having a high content of linoleic acid. They are used for topical application and provide the skin with essential polyunsaturated fatty acids, which support the formation of ceramide 1 and with choline which is a part of the natural moisturizing factor (NMF). In a clinical study it was proven that these liposomes have cosmetic properties, i.e., improve skin appearance, like inducing wrinkle reduction and an increase in skin smoothness. Additionally, these liposomes exert pharmaceutical effects, like decreasing of efflorescence in the acne treatment (Blume and Teichmüller 1997; Ghyczy et al. 1996). Recent approach in modeling transdermal drug delivery through the skin is the development of two ultraflexible vesicular carriers —ethosomes and Transfersomes®.

Sterically stabilized liposomes (long-circulating liposomes, Stealth® liposomes) are composed of hydrophilic polymer polyethylene glycol (PEG), attached covalently to the outer surface. PEG coating inhibits protein adsorption and opsonization of liposomes, thereby avoiding or retarding liposome recognition by the reticuloendothelial system (Lasic et al. 1991).

Table 2.2 defines the terms that are most commonly found in literature and relate them to different types of liposomes.

Table 2.2 Classification of commonly known lipid vesicles according to their structures and/or preparation

Identification	Definition
Archaeosomes	Archaeosomes are vesicles consisting of archaebacterial lipids which are chemically distinct from eukaryotic and prokaryotic species. They are less sensitive to oxidative stress, high temperature, and alkaline pH (Krishnan et al. 2000; Conlan et al. 2001)
Cationic liposomes	Composed of cationic lipids. Fuse with cell or endosome membranes; suitable for delivery of negatively charged macromolecules (DNA, RNA); ease of formation, structurally unstable; toxic at high dose, mainly restricted to local administration
Cochleates	Cochleates are derived from liposomes which are suspended in an aqueous two-phase polymer solution, allowing the logic partitioning of polar molecule-based structures by phase separation. The liposome containing two-phase polymer solution treated with positively charged molecules such as Ca2+ or Zn2+ form sacochleate precipitate of a particle dimension less than 1 μm (Gould-Fogerite et al. 1998)
Conventional liposomes	Composed of neutral or negatively charged phospholipids and cholesterol. Rapid and saturable uptake by reticuloendothelial system; short circulation half-life, dose-dependent pharmacokinetics
Dendrosomes	Nontoxic, neutral, biodegradable, covalent or self-assembled, hyperbranched, dendritic, spheroidal nanoparticles which are easy to prepare, inexpensive, highly stable (Sarbolouki et al. 2000)
Dried reconstituted vesicles (DRV)	By this preparation technique, small, "empty" unilamellar vesicles, containing different lipids or mixtures of them, are prepared. After mixing those SUVs with the solubilized drug, dehydration is performed. By addition of water, rehydration leads to the formation of large quantities of rather in homogeneous, multilamellar vesicles which need further processing (Gregoriadis et al. 1987)
Ethosomes	Ethosomes are composed of high amounts of ethanol (45 % v/v) and a low lecithin (2 % w/v) concentration, which provide an ethosome suspension with mean size of approximately 100 nm. Ethosomal systems are much more efficient at delivering to the skin, in terms of quantity and depth, than either conventional liposomes. Ethosomal systems composed of soy phosphatidylcholine and about 30 % of ethanol were shown to contain multilamellar vesicles by electron microscopy (Touitou et al. 2000; Godin and Touitou 2003)
Immunoliposomes	Conventional or stealth liposomes with attached antibody or recognition sequence
Immunosomes	Immunosomes are prepared by the anchorage of glycoprotein molecules to preformed liposomes. Under the electron microscope, immunosomes look like homogenous spherical vesicles (50–60 nm) evenly covered with spikes. Immunosomes have structural and immunogen characteristics closer to those of purified and inactivated viruses than any other forms of glycoprotein lipids association (Perrin et al. 1985)
Immune stimulating complex (ISCOM)	ISCOMs are spherical, micellar assemblies of about 40 nm. They are made of the saponin mixture Quil A, cholesterol, and phospholipids. They contain amphiphilic antigens like membrane proteins. ISCOMs already have a built-in adjuvant, quillaja saponin, which is a structural part of the vehicle (Kersten and Crommelin 2003)
Invasomes	Liposomes containing besides PC also ethanol and terpenes (Dragicevic-Curic et al 2008)
Lipoplexes	Liposomes, comprising cationic and neutral lipids, which are able to form complexes with negatively charged pDNA. They are efficient carriers for cell transfection but have certain drawbacks due to their toxicity. These toxic effects may result from either cationic lipids or nucleic acids (Khalil et al. 2006; Audouy and Hoekstra 2001)
Long-circulating liposomes	See stealth liposomes. Composed of neutral, high transition temperature lipid, cholesterol, and 5–10 % of PEG-DSPE. Hydrophilic surface coating, low opsonization, and thus low rate of uptake by RES long-circulating half-life (40 h); dose-independent pharmacokinetics (Lasic et al. 1991; Woodle and Lasic 1992; Moghimi and Szebeni 2003)
LUVETs	LUVETs are large unilamellar vesicles prepared by extrusion technique, mainly performed with high-pressure systems (Mayer et al. 1986)
Magnetic liposomes	Composed of PC, cholesterol, and small amount of a linear chain aldehyde and colloidal particles of magnetic iron oxide. Can be made use by an external vibrating magnetic field on their deliberate, on-site, rapture, and immediate release of their components

Table 2.2 (continued)

Identification	Definition
Marinosomes	Liposomes based on a natural marine lipid extract containing a high polyunsaturated fatty acid (PUFA) ratio (Moussaoui et al. 2002)
PLARosomes	Liposomes with phospholipid alkylresorcinol (PLAR), which improves the stability and the entrapment of the liposomal drug formulation (Gómez-Hens and Fernández-Romero 2006)
pH-sensitive liposomes	Four basic classes of pH-sensitive liposomes have been described The first class combines polymorphic lipids, such as unsaturated phosphatidylethanolamines, with mild acidic amphiphiles that act as stabilizers at neutral pH; suitable for intracellular delivery of weak base and macromolecules The second class includes liposomes composed of lipid derivatives resulting in increased permeability to encapsulated solutes A third class utilizes pH-sensitive peptides or reconstituted fusion proteins to destabilize membranes at low pH The fourth class uses pH-titratable polymers to destabilize membranes following change of the polymer conformation at low pH (Drummond et al. 2000)
Polymerized liposomes	Polymerized phosphatidylcholine vesicles (35–140 nm) have been synthesized from lipids bearing one or two methacrylate groups per monomer. Compared to nonpolymeric analogues, these vesicles exhibited improved stability and controllable time-release properties (Regen et al. 1981)
Proliposomes	Proliposomes are defined as dry, free-flowing particles that immediately form a liposomal dispersion on contact with water
Proteasomes	Liposomal vesicles of bacterial origin, with a protein to lipid ratio higher than that achieved with purified protein incorporated in liposomes. Vesicles were solubilized, followed by ammonium sulfate precipitation and dialysis against detergent buffer. Proteins and peptides are noncovalently complexed to the membrane, making them highly immunogenic (Lowell et al. 1988)
Reverse-phase evaporation vesicles (REV)	Vesicles are formed by evaporation of oil in water emulsions resulting in large unilamellar liposomes (Szoka and Papahadjopoulos 1987)
Stealth liposomes	Liposomes sterically stabilized by covalent attachment of hydrophilic polymers, mainly polyethyleneglycols (PEGylated liposomes) to the bilayer, which show prolonged circulation half-lives and can evade interception by the immune system. PEG coating inhibits protein adsorption and opsonization of liposomes, thereby avoiding or retarding liposome recognition by the reticuloendothelial system (RES). These PEG-coated liposomes are also referred to as sterically stabilized or stealth liposomes. The PEG-stabilizing effect results from local surface concentration of highly hydrated groups that sterically inhibit both hydrophobic and electrostatic interactions of a variety of blood components at the liposome surface (Allen et al. 1991; Klibanov et al. 1990)
Temperature-sensitive liposomes	Such liposomes have been prepared using lipids which undergo a gel-to-liquid crystalline phase transition a few degrees above physiological temperature. The most suitable lipid is dipalmitoylphosphatidyl choline. Temperature-sensitive liposomes are considered to be a promising tool to achieve site-specific delivery of drugs They release the entrapped content at the target cell surface upon a brief heating to the phase transition temperature of the liposome membrane (Kono 2001; Needham and Dewhirst 2001)
Transfersomes	Transfersomes are elastic, very deformable vesicles which consist of PC in combination with an edge active surfactant like sodium cholate, which softens the membrane of the vesicle and makes the bilayer much more flexible. The strong hydrophilicity and the vesicle's extreme ability to deform enable the transfersome to temporarily open the pores through which water normally evaporates between the cells. Such newly activated intercellular passages can accommodate sufficiently deformable vesicles maintaining their integrity but changing their shape to fit the channel. Along these said pathways in the horny layer, transfersomes reach regions of high water content in the deeper skin layers (Paul and Cevc 1995). Transfersome is a term registered as a trademark by IDEA AG, Germany
Virosomes	Liposomal formulations with viral envelope proteins anchored in their lipid membrane (Glück 1999)

Partly from Wagner and Vorauer-Uhl (2011)

2.7 Methods for Characterization, Quality Control, and Quantification of Percutaneous Penetration of Liposomes

The use of liposomes requires the control of several features, such a size distribution, composition, encapsulation efficiency (EE), and chemical stability. The tests, including lipid integrity, particle size, morphology of liposomes, phase transition temperature, and drug release rates, are proposed for the stability study of liposomes.

Chemical analysis of liposomes sheds light on the purity of lipids and can quantitatively reveal the absence or presence of degradation products formed by lipid hydrolysis or peroxidation. The hydrolysis of lipids leads to the formation of lysolipids. As the concentration of lysolipids increases in a given solution of liposomes, the bilayer structure of the liposome is eventually disintegrated and the undesirable formation of micelles takes place. The hydrolysis of lipids depends on the pH of the media employed. Reported data indicate that, at pH 6.5, lipid hydrolysis is minimal (Lasic and Templeton 1996).

Lipids with unsaturated acyl chains tend to oxidize due to the presence of oxidizing agents present in the media. This process results in fragmented acyl chains which contribute to the destabilization of the bilayer structure of the liposomes. It is, therefore, very important to provide an optimum pH range as well as the presence of antioxidants in liposome products in order to reduce hydrolysis and oxidation. Ultraviolet (UV) spectra of an ethanolic solution of a lipid can reveal if the lipid is oxidized. An absorbance at 230 nm will suggest the presence of conjugated dienes, while absorbance between 270 and 280 nm will indicate the presence of conjugated trienes, and this suggests that degradation of the lipid has occurred (Mhashilkar et al. 2001). Other commonly used methods of lipid analysis include thin-layer chromatography (TLC) (Oberholzer et al. 1995), high-performance liquid chromatography (HPLC) (Arts et al. 1997), gas chromatography (Müller et al. 2004), nuclear magnetic resonance (NMR) spectroscopy, and differential scanning calorimetry (DSC).

The size of liposomes is commonly characterized by two different methods: light scattering and transmission electron microscopy (TEM). Light scattering, including low-angle light scattering and photon correlation spectroscopy (PCS), is commonly applied for generating specifications of liposomes in the cosmetic and pharmaceutical industries. For SUV or LUV types of liposomes, the PCS method is preferred over the low-angle light scattering method. Further, TEM (negative staining method and cryo-TEM) images allow accurate measurement of the liposome size, while the freeze fracture TEM sheds light on lamellarity and surface morphology of liposomes.

Lipid thermal behavior is studied using a differential scanning calorimeter.

A new method to determine the integrity and intactness of liposomes in final formulations is electron spin resonance (ESR) spectroscopy. First of all, liposomes are labeled with an ESR-active probe (chemically similar to PC), and afterward ESR spectra of formulations are taken. By computer simulation the degree of stability or degradation can be calculated. An excellent stability could be detected in aqueous systems. In w/o emulsions the stability of liposomes varied between 50 and 100 % (over a time period of 8 weeks) depending on the emulsifiers used. The stability less than 50 % was found in a w/o formulation, and a total breakdown could be observed in shampoos (Magdassi 1997).

Electron paramagnetic resonance (EPR) has been used to investigate the influence of liposome size on the transport of substances and the interactions of liposomes with the skin (Šentjurc et al. 1999, 2004).

Most of the methods described to determine the percentage of drug carried by liposomes (i.e., encapsulation efficiency, EE) involve removal of the non-encapsulated drug using size exclusion chromatography (SEC) (Kaiser et al. 2003; Junping et al 2000), centrifugation (Moribe et al. 1998), dialysis, and filtration (Wu et al. 2004). Proton NMR spectroscopy is an alternative technique that allows the determination of liposomal

EE without physical separation of entrapped and non-entrapped drug (Zhang et al. 2004).

The development of analytical methods to control the effectiveness of liposomal delivery systems (LDSs) runs parallel to the development of these LDSs (Koppenhagen et al. 1998). Although many methods have been described for the control of liposomal drug formulations, most of them measure only total drug concentration in the sample and do not distinguish between free and entrapped drug, which would be desirable to know to establish the real behavior of these formulations.

2.7.1 Methods for Separation Liposomal and Non-liposomal Drug Forms

Solid-phase extraction (SPE) is of great interest in the separation of liposomal and non-liposomal drug forms. Separation is based on the property of liposomes to cross reversed-phase C18 silica gel cartridges without being retained, while a non-liposomal drug is retained on the stationary phase (Deshpande et al. 2010).

Size exclusion chromatography (SEC) is a simple and powerful technique for the investigation of encapsulation, insertion/interaction of substances from small solutes (ions, surfactants, drugs, etc.) up to large molecules (proteins, peptides, and nucleic acids) in liposomes. The drug retention capacity of the liposomes is usually determined. SEC is widely used to narrow the size distribution. For example, Sepharose 2B, 4B Sephacryl S-1000, and high-performance exclusion gels of the TSK-PW series are suitable for separating small unilamellar vesicles from larger ones using SEC. The potentiality of SEC is strongly improved by using a HPLC system associated to gel columns with a size selectivity range allowing liposome characterization in addition to particle (Lesieur et al. 1993; Lundahl et al. 1999; Grabielle-Madelmont et al. 2003).

Thin-layer chromatography (TLC) is an old method for characterization and investigation of chemical stability of phospholipide.

High-performance liquid chromatography (HPLC) has been widely applied for the determination of drugs in liposome formulations (Chimanuka et al. 2002; Ferdous et al. 1997; Rossi et al 2004; Grohganz et al. 2004). A limitation of this method is that it involves the disruption of liposomes, measuring total drug in the sample and not the entrapped drug.

Capillary electrophoresis (CE) with chemiluminescence detection has been used for the characterization of liposomes in order to study different properties, such as homogeneity, trapped volume, stability, and permeability (Tsukagoshi et al. 1998; Griese et al. 2001).

Future innovations in analytical techniques will be oriented toward the development of new approaches to provide online and in situ information on the penetration of drugs into the skin.

There have been several methods reported in literature for the quantification of the percutaneous penetration enhancement. These include diffusion experiments (Du Plessis et al. 1994), visualization by electron microscopy (Hofland et al. 1995), and micro dialysis (Schnetz and Fartasch 2001). Micro dialysis and diffusion experiments provide information about the amount and the rate of drug penetration of the model compound, but do not give any information about the physiological effects of the model drug on cells and lipid organization. The visualization by electron microscopy provides detailed information about the structure of the cells and lipid organization in the skin, but does not provide information about the penetration pathways. Other techniques used are fluoromicrography (Yarosh et al. 1994) and confocal laser scanning microscopy (CLSM). Fluoromicrographs of the skin treated with fluorescently labeled liposomes demonstrated that the fluorescent marker remained in the SC or penetrated deeper in the epidermis mainly along the follicle. A disadvantage of fluoromicrography is that the tissue needs to be (cryo)fixed, which may change skin lipid organization or may result in redistribution of the marker (Shotton and White 1989). CLSM provides information about the localization and the permeation pathway of a fluorescent model compound in the tissue. The major advantage of CLSM is that the distribution of the fluorescent model compound in the sample can be visualized

without cryofixing or embedding the tissue. However, in the case of penetration studies with liposomes, CLSM does not provide information about the permeation of the entire liposome, but only about the penetration of the fluorescent label (Õan Kuijk-Meuwissen et al. 1998).

2.8 The Stability of Liposomes

The stability of liposomes involves the stability of the particles and all their components, including the encapsulated substance. Liposome stability can be subdivided into physical, chemical, and biological stabilities, which are all interrelated. Generally, the shelf-life of liposomes is determined by the physical and chemical stability. By optimizing the size distribution, pH, and ionic strength, as well as the addition of antioxidants and chelating agents, liquid liposome formulations can be stable (Mojović et al. 1996a, b; Šiler-Marinković et al. 1996).

2.8.1 Physical Stability

Physical instability of the liposomes is expressed as a fusion of vesicles, aggregation, and encapsulation capacity reduction. The consequence of fusion is the loss of encapsulated material. By proper selection of lipid components as well as storing conditions for liposomes, an optimum membrane permeability can be obtained. Risk of fusion can be reduced by applying negatively charged phospholipids, which increase the density of charge on the surface of the membrane of liposomes. Addition of cholesterol reduces the permeability of the membrane when it is in liquid-crystalline thermodynamic state. Some authors believe that cholesterol can autoxidize over time, leading to loss of encapsulated material (Šobić and Šiler Marinković 2002).

The high membrane permeability for water-soluble molecules is the cause of the sensitivity liposomes to the osmotic pressure. Larger liposomes (MLV and LUV) are more sensitive, and they can swell on the high osmotic pressure. At the critical value of the osmotic pressure, liposomes may rupture.

2.8.2 Chemical Stability

Chemical instability of the liposomes is reflected in the oxidation of unsaturated fatty acids present in the lipid bilayer, lipid hydrolysis, and degradation of the active components, which may lead to disruption of liposome membranes and leaking of encapsulated materials.

The oxidation rate depends on the nature of the phospholipids and the presence of antioxidants and prooxidants. Oxidation can be slowed down using hydrogenated phospholipids as starting material, avoiding contact with oxygen, reducing the exposure to light and heat (Šiler-Marinković and Mojović 1996).

Liposomes are susceptible to enzymatic and chemical hydrolysis, but it is not an important cause of instability. Enzymatic hydrolysis of lipids is catalyzed by lipolytic enzymes. Lipases of vegetable origin operate normally under acidic conditions, while animal lipases act in an alkaline environment. The rate of enzymatic hydrolysis was higher for lipids with unsaturated fatty acids. Chemical hydrolysis is, in contrast to the enzyme hydrolysis, less controlled. This process depends on pH, and it is slowest at pH 6.5 for most phospholipids. In order to stop the hydrolytic changes in the liposomes, dispersions can be prepared with Tris-buffered saline or phosphate-buffered saline of certain pH. Temperature affects the rate of hydrolysis which is much faster at temperatures higher than 25 °C. The reason may be the activation of lipases, which are present in the raw materials of natural origin.

2.8.3 Microbiological Stability

Liposomes are an excellent medium for bacterial growth. Liposomes used for cosmetic purposes are not sterilized, but adequate sterility can be achieved by the passage of liposomes through up to millipore filters (400 nm), and after that, dispersions are protected with preservatives. The preservatives may interact with double-layer membranes, which are manifested by changes in Tm. This phenomenon is most pronounced in propylparaben and must be taken into account in

the formulation of liposomal dermatics (Wallhaeusser 1995). Ethanol, as a possible preservative, has also limitations, because its addition increases the average diameter of liposomes. Addition of 10 % ethanol increases the average diameter of liposomes by about 20 %, while the addition of 30 % ethanol causes an increase in the mean diameter of about two times (Kirjavainen et al. 1997; Maitani et al. 2001).

2.9 Liposomes as Skin Drug Delivery Systems

Delivery, as we know, is the process of transporting the right chemical to the right location at a relevant concentration for a sufficient period of time. Many factors govern the delivery of active ingredients from topically applied formulations into the skin. These factors include type of formulation, the lipophilicity of the component, the size of the molecule, presence of penetration enhancers, and physical state of the stratum corneum (SC).

Many studies showed significantly higher absorption rates and greater pharmacological effects for drugs encapsulated in liposomes, as compared to conventional topical formulations (Michel et al. 1992; Mezei and Gulasekharam 1980; Cevc 1996; Rownicka-Zubik et al. 2012).

There are three different skin penetration routes for applied drugs. These include the intercellular route, the transcellular route, and follicular penetration. In the past, the intercellular route has been considered the predominant pathway of skin penetration. Hair follicles and sweat glands account for approximately only 1 % of the skin surface, and, therefore, the follicular route was not previously considered to represent a significant route of penetration. Surprisingly, however, recent in vitro and in vivo studies suggest that the follicular route is also important for skin penetration (Lademann et al. 2001). However, the mechanisms of action of liposomes in inserting active ingredients into the skin remain controversial. But, in the last few years, some authors highlighted important factors that influence the penetration of active ingredients encapsulated in liposomes. Several factors, such as total lipid concentrations and composition, lamellarity, charge of liposomal surface, and mode of application, have been proven to influence drug deposition into the skin layers (Cevc and Blume 1992; Weiner et al. 1989). The experimental design may have also some effects on the recorded action. Accordingly, it is not possible to describe a general mode or mechanism of action of liposomes (acting as skin drug delivery systems) with skin, and in each situation, a detailed description of the formulation and experimental design has to be considered (Tikshdeep et al. 2012).

Four alternative mechanisms have been suggested for liposomes acting as delivery systems (Fig. 2.6) (El Maghraby et al. 2005, 2006). The first mechanism (A) represents a free drug operation, whereby molecules are initially released from liposomes and they independently permeate through the skin. In this case, vesicles can be considered only as carriers that can control drug release. The second mechanism (B) suggested a possible penetration enhancement effect, because lecithin enchases transdermal delivery by lowering the permeability barrier of the skin. The third mechanism (C) is the adhesion of liposome lipids onto the skin surface, with possible fusion or mixing with the lipid matrix of the SC. It is supposed that once in contact with the skin, some budding of liposomal membrane might occur (Schaller and Korting 1996; Vrhovnik et al. 1998). This could cause a mixing of the liposome bilayers with intracellular lipids in the SC, which may change the hydration conditions and thereby the structure of lipid lamellae of the SC. This may enhance the permeation of the lipophilic drug into the SC and ease the diffusion of hydrophilic drugs into the interlamellar spaces. The concept of intact vesicular skin penetration (D) is not recent and was suggested in the first reports on liposomes as skin drug delivery systems. It was difficult to conceive that large lipid vesicles could penetrate the densely packed SC. Consequently, many research groups have tested this hypothesis, and electron micrography showed the presence of few intact liposomes in the dermis (Foldvari et al. 1990). Although the applied formulation included large multilamellar vesicles (LMLVs), most of the

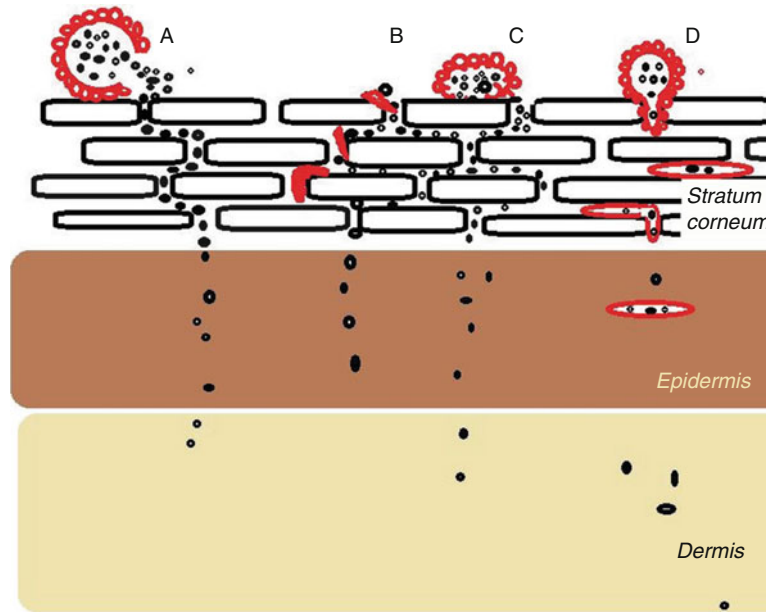

Fig. 2.6 Interaction of the liposomes and skin (El Maghraby et al. 2006)

liposomes detected in the dermis were unilamellar (300–500 nm) with some LMLVs. The authors proposed that liposomes could penetrate the epidermis carrying the drug into the skin and that the smaller nano-aggregates could have come from LMLVs that lost their external bilayers during penetration. The liposomes could be adsorbed intact on the skin surface before penetration, with a possibility that some vesicles might rupture. It may be possible that some vesicles, which are deformable enough, will pass the SC as intact structures (Cevc and Blume 1992; Cevc et al. 2002) or may accumulate in the channel-like regions in the SC (Honeywell-Nguyen et al. 2004) depending upon their compositions. It is reported by several authors that the high elasticity of vesicles could result in enhanced drug transport across the skin as compared to vesicles with rigid membranes. In any case, liquid-state, flexible liposomes showed greater skin penetration than those in a gel state, and small-sized and unilamellar vesicles seem to result in a higher degree of skin penetration (Blume et al. 2003; Fresta and Puglisi 1996; Verma et al. 2003; Honeywell-Nguyen et al. 2004).

The application conditions can also influence the penetration kinetics. The flexible vesicles work more efficiently under nonocclusive application. The driving force for the transport of vesicles into the skin is the trans epidermal osmotic gradient, and nonocclusive application is the key to create it (Cevc and Blume 1992). The driving force for the movement of flexible liposomes is generated by the hydration gradient across the skin, which builds up because of the different water content in the SC and stratum granulosum (SG), i.e., the content varies from 15 to 20 % in the SC to 70 % in the SG (Fresta and Puglisi 1996). When the flexible liposomes are applied onto the skin and allowed to dry, the vesicles as hydrophilic entities/structures are attracted by the moisture in the epidermis, and due to their flexibility, they penetrate the skin. After reaching in the local area, a liposome can interact with the skin cell by any of the following methods (Anwekar 2011):

- Endocytosis by phagocytic cells of the reticuloendothelial system (RES) such as macrophages and neutrophils
- Adsorption to the cell surface either by nonspecific weak hydrophobic or electrostatic forces or by specific interaction with cell surface components
- Fusion with the plasma cell membrane by insertion of lipid bilayer of liposome into the

plasma membrane with simultaneous release of liposomal contents into the cytoplasm
- Transfer of liposomal lipids to cellular or subcellular membrane or vice versa without any association of the liposome contents

It is often difficult to determine what mechanism is operative and more than one may operate at the same time.

To continue the action of drugs to a particular site in the body, the general approach is to deposit drug-bearing liposome directly into the site where therapy is desired. Since liposomes are large and do not easily cross epithelial or connective barriers, they are likely to remain at the site of local administration. The liposomes would then slowly released into the target site or perhaps create a local drug level higher than the systemic level. Alternatively the drug-loaded liposomes might interact directly with cells in the target site, without producing drug release. The goal of this approach is to maximize the amount of effective drug at the target site, while minimizing the drug levels at other sites and thus decreasing systemic toxicity (Tatsuhiro 2002).

2.10 Application of Liposomes as Dermal Delivery Systems

Liposomes are efficient delivery systems for drugs. They can act as drug carriers controlling release of the therapeutic agent. Alternatively they may provide a localized depot in the skin so reducing the amounts of drug permeating through the skin, thus minimizing systemic effects. They may also provide targeted delivery to skin appendages. Further, vesicles can enhance transdermal drug delivery, increasing systemic drug concentrations. In addition, the use of liposomes in nanocosmetology also has many benefits, including improved penetration and diffusion of active ingredients, transport of active ingredients, extended release time, greater stability of active ingredients, reduction of unwanted side effects, and high biocompatibility. Liposomal preparations reduce the skin roughness because of their interaction with the corneocytes and with the intercellular lipids resulting in skin softening and smoothening (Pierre and dos Santos Miranda Costa 2011).

Liposomes can be loaded with different hydrophilic, lipophilic, or amphiphilic *cosmetic actives*. Examples include vitamins, α-hydroxy acids, UV filters, ceramides, unsaturated fatty acids, growth factors, botanical extracts, and antioxidants (Blume 2008). In these cases, formation of gels containing the corresponding active-loaded liposomes is easily achieved. There is a considerable interest in the use of liposomes for products that retard premature aging of skin, or prevent photoaging. Antioxidants along with natural botanical extracts are commonly used in antiaging products, and encapsulation of vitamins in liposomes has been shown to enhance their stability. Encapsulation and release studies of personal care actives like α-hydroxy acids, β-hydroxy acids (salicylic acid) (Perugini et al. 2000), retinoids (Li and Lishko 2001), and ascorbic acid (Fočo et al. 2005) have been published in the past. The first liposomal cosmetic product to appear on the market was the antiaging cream "Capture" launched by Dior in 1986. Topical liposomal preparations containing encapsulated compounds have recently attained commercial value.

Glycolic acid, excellent as an exfoliative agent and moisturizer used in cosmetic products, has an irritant effect when applied on the skin. Loading glycolic acid into the liposomes showed the advantage of liposomes in reducing side effects (e.g., irritation) of glycolic acid. The results obtained showed that liposomes are suitable to modulate glycolic acid release and thereby its irritation potential and that the best condition to achieve this control is obtained by the liposomal systems in which glycolic acid/lipid molar ratio is 5:1. Further significant release control is obtained by addition of chitosan into the liposome (Perugini et al. 2000).

Retinoids, drugs useful in the treatments of acne, psoriasis, and many tumors, were recently shown to have reduced side effects when applied entrapped in liposomes. Decreased skin burning and increased drug stability after exposition to light was demonstrated by the use of this delivery

system (Li and Lishko 2001; Trapasso et al. 2009). Cationic liposomes consisting of double-chained cationic surfactants, PC, and retinoic acid were found to increase delivery of retinoic acid about twofold suggesting the potential of cationic liposomes' use for the intradermal delivery of lipophilic drugs like retinoic acid (Kitagawa and Kamasaki 2006).

Stability and low pH value of *ascorbic acid* are the difficulties for formulating effective cosmetic products containing these actives. In the study of Fočo et al. (2005), two types of multilamellar vesicles, one from non-hydrogenated and the other from hydrogenated soybean lecithin, containing cholesterol, were prepared for the encapsulation of sodium ascorbyl phosphate (SAP). It was shown that liposome carriers enabled high SAP penetration into SC. The difference in liposome composition did not have any significant effect on the penetration profile of SAP which was much more influenced by the concentration of the drug. At higher concentrations of SAP, the absolute penetrated amount of SAP was higher. As to the stability of SAP in liposome formulations, it was much more influenced by the storage temperature than by liposome composition.

Liposomes with encapsulated lipolytic actives are used for the treatment of *obesity*. Recently, Tholon et al. have prepared so-called slimming liposomes from soy phospholipids and extract of *Centella asiatica*, L-carnitine, and other actives. Their in vivo data indicate that a daily topical application of gel with 3 % slimming liposomes reduced thigh circumference by 10 mm in 28 days (for >20 % of the subjects) (Tholon et al. 2002).

The treatment of many dermatological diseases by topical application of liposomal formulations is expected to be more efficient, because a significant concentration of the drug is retained in the living epidermis and dermis. Triamcinolone, methotrexate, hydrocortisone, and diclofenac are some of the drugs encapsulated in the liposomes with promising results. Liposomes with methotrexate reduced the percutaneous drug absorption, while the drug retention in the skin was two- to threefold higher than when the free drug form was used (Betz et al. 2001; Schmid and Korting 1994; Yarosh 2001; Samad et al. 2007).

Liposomal preparations were found superior in the treatment of acne vulgaris compared with conventional preparations including alcoholic lotions (Meybeck 1992; Ghyczy et al. 1996). A double-blind clinical study was conducted to assess the safety and efficiency of liposome-encapsulated 1 % clindamycin solution versus 1 % clindamycin solution (Klimicin® T, Lek). On the basis of the clinical trial, it may be concluded that liposome-encapsulated 1 % clindamycin solution was therapeutically superior over conventional 1 % clindamycin solution in the treatment of acne vulgaris (Honzak and Sentjurc 2000).

The transdermal route for *local anesthetics* has advantages over injection pathway, such as improving patient compliance, providing continuous drug delivery, and avoiding side effects including hematoma and nerve damage. Poor permeability and slow penetration rate into the skin of existing topical anesthetics, however, have significantly limited their clinical applications. An alternative delivery strategy used to increase penetration of topical anesthetics is the incorporation of the drug into the liposomes (Eichenfield et al. 2002; Lener et al. 1997; Miler et al. 2004). The potential of liposome-encapsulated local anesthetics to provide topical anesthesia to intact skin was investigated. Using the pin-prick assay, prolonged anesthesia from a tetracaine or a lidocaine liposome formulation was shown, whereas a cream control formulation was ineffective. The improved anesthetic effect after vesicle delivery was probably due to improved skin accumulation (Gesztes and Mezei 1988). Vesicles provided even stronger and deeper anesthesia relative to a commercial eutectic mixture of local anesthetics EMLA (APP Pharmaceuticals LLC, Schaumburg, Il.) 2.5 % lidocaine and 2.5 % prilocaine in man (Fisher et al. 1998). Biozone Labs, Pittsburg, CA,USA, produces a liposomal formulation with lidocaine available at the market under the commercial name ELA-Max ™. ELA-Max is a registered trademark of Ferndale Laboratories, Inc.

Dithranol is one of the drugs used in the topical treatment of psoriasis, but it has irritating, burning, staining, and necrotizing effects on the skin. The entrapment of drugs in liposomes will

reduce the dose-dependent side effects, like irritation and staining. The entrapment efficiency of dithranol in the liposomes was optimized by altering the proportion of PC and cholesterol. The in vitro permeation study using mouse abdominal skin showed significantly enhanced permeation with vesicles as indicated by the flux of dithranol from liposomes (23.13 μg/cm^2/h) and niosomes (7.78 μg/cm^2/h) as compared with the cream base (4.10 μg/cm^2/h) (Agarwal et al. 2001).

The deposition of *hydrocortisone* into the human skin was significantly higher when liposomes were used compared with the ointment form. The blanching effect of hydrocortisone paralleled the deposition results, with the vesicles producing a greater effect than the ointment even when urea was incorporated into this base (Wohlrab et al. 1992).

Liposomal delivery of *local antibiotics* may be clinically useful in surgical wound prophylaxis, and the advantages include the achievement of adequate local antimicrobial concentration at the time of operation as well as increased efficacy of the antibiotic. The liposomal delivery of local antibiotics in this model of surgical wound infection reduced the number of organisms more effectively than the applied free drug. Treatment of contaminated surgical wounds is often complicated by the failure of local or systemic antibiotic treatment and prophylaxis. Locally administered liposome-encapsulated antimicrobials may offer advantages over free antibiotics, including an increase in efficacy, ease of administration, and safety. The local delivery of antibiotics by liposomes may also have advantages in other areas such as oncology, wound healing, immunology, and cellular biology. The therapeutic advantages, as well as the absorption and distribution of locally administered liposome-encapsulated antibiotics, were compared with those of locally applied unencapsulated antibiotics in a contaminated wound model. Liposomes can potentially alter toxicity and target drug delivery to specific sites (Šiler-Marinković et al. 1997; Honzak and Sentjurc 2000).

The successful targeting of actives via hair follicles using liposome delivery systems is an "open door" for cosmetic applications as well. Cotsarelis and his group at the University of Pennsylvania have investigated gene delivery to hair follicles using liposomes (Domashenko et al. 2000; Gupta et al. 2001). Hoffman has also reported on the liposomal targeting of hair follicles (Hoffman 1998). These studies have shown encouraging results and may become a practical way of treating hair disorders by means of DNA treatment. Introducing hair growth promoters or hair growth retardants through liposomal formulations may also be a practical approach.

Liposomes have also been used in the treatment of hair loss. *Minoxidil*, a vasodilator, is the active ingredient that claims to prevent hair loss. It is formulated in liposomes to improve the flux of contents through the skin (Bhushan 2008). Minoxidil sulfate in PG-coated liposomes is also marketed as Nanominox-MS (Sinere, USA). Liposomes with *progesterone*, on the other hand, have been used to reduce the rate of hair growth in idiopathic hirsutism (Ganesan et al. 1984).

A formulation with *melanin*-encapsulating liposomes in a spray form (Lipoxome®, Dalton Medicare B.V., the Netherlands) delivers melanin selectively to the hair follicle and the hair shaft to stain hair follicles of people with bold, white, or gray hair (Sand et al. 2007).

Conclusion

As a conclusion, liposomes offer great value in dermal/transdermal drug delivery, and recent advances of liposomes appear to have generated increased therapeutic potential. Alteration in their composition and structure results in vesicles with better properties. Flexible liposomes are one such advance with claims of enhanced transdermal drug delivery.

Liposomes continue to be an area of research to be further explored for a better understanding and characterization of the transport path and interaction with the skin. Most of the researchers agree that the use of liposomes has many benefits, including improved penetration of active ingredients, selective transport of active ingredients, longer release time, greater stability of active ingredients, reduction of unwanted side effects, and high biocompatibility.

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Deformable (Transfersome®) Vesicles for Improved Drug Delivery into and Through the Skin

3

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3.1 Introduction

The skin (*cutis*) is the largest organ in a mammalian body. It is also the best and the easiest accessible body part. Transcutaneous application is therefore an attractive option for drug delivery. The problem with the approach is that most drugs cannot diffuse (*permeate*) well through an intact skin. This is owing to the special anatomical, biochemical and physiological properties of the organ. The primary reason is the outermost, 8–30 μm thin, skin region, the horny layer or the stratum corneum, which forms one of the best natural barriers to transport (Fig. 3.1, left).

To overcome the skin's primary (i.e., permeability) barrier, various methods had been designed. The most common and invasive of them relies on injectors, including but not limited to microneedles. It is now possible to penetrate the mammalian skin with the specially engineered tiny needle(s) packs to a depth of just a few ten micrometres (Cevc and Vierl 2010). Such hollow needles are useful for drug injection, whereas the solid needles carry drug cargo on their surface (Cleary 2011; Escobar-Chávez et al. 2011). In either case, one creates a limited number of pores through the cells in the outer skin (*epidermis*) (Prausnitz 2004). Epidermis breaching with high-velocity particles or droplets, with high voltage electric or thermal pulses, or with sonoporation is also potentially useful for drug delivery but is less well controlled (Cevc and

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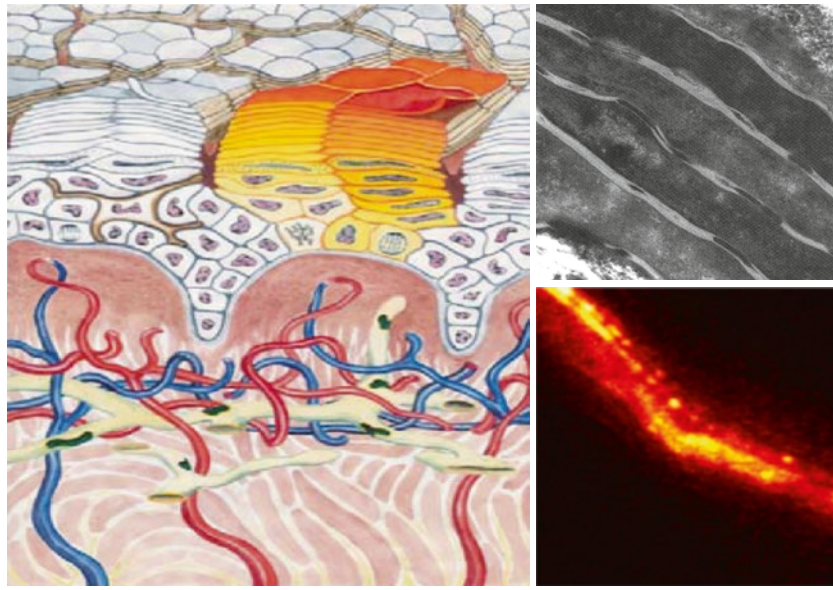


Fig. 3.1 *Left*: schematic representation of a mammalian skin, including the outer epidermis, full of tightly packed cells (corneocytes), which is separated from the blood vessel-rich dermis by the basal cells layer. Cells within the outermost region, the stratum corneum, which form the skin's primary barrier, are grouped in clusters, surrounded by 'furrows' or 'clefts' (with a lower permeation resistance). *Upper right*: contacts between the flat and vertically stacked corneocytes within the stratum corneum are sealed by intercellular lipids (dark lines), but water can nonetheless evaporate between cells through hydrophilic conduits. *Lower right*: fluorescently labelled deformable vesicles can spontaneously widen and then trespass the hydrophilic conduits, driven by the natural transepidermal hydration gradient, simultaneously delivering exogenous material from skin surface toward skin depth (Based on Cevc et al. 2003 and Schätzlein and Cevc 1998)

Vierl 2010). The same holds true for the stratum corneum abrasion with mechanical means or with radio-frequency waves (Levin 2008).

Despite their potential benefits, none of the above listed methods is broadly used to date (Prausnitz and Langer 2008). The reasons include skin irritation, inconvenience and/or high cost of use. Technical complexity and limited deliverable drug dose also play a role. Consequently, no marketed pharmaceutical product for transcutaneous drug delivery involves a modern technological solution and/or contains a large (<400–500 Da) molecule. Indeed, all such products rely on the oldest non-invasive procedure for lowering the skin permeability barrier: chemical skin permeability enhancement by partial extraction and/or fluidisation of the stratum corneum lipids (Hadgraft and Lane 2006). This method fails to improve large molecule delivery across intact skin, however, owing to the inability of such molecules to partition into and diffuse across the skin lipidic barrier.

If one merely wishes to restrict delivery of an epicutaneously administered drug to peripheral tissue below the administration site, one faces another problem. The small molecules that have diffused across the stratum corneum reach the extensive network of cutaneous blood capillaries in the lower skin region (*dermis*), with a total area that exceeds the skin surface area between two- and fivefold (Cevc and Vierl 2007). The cutaneous blood flow thus effectively acts as the skin's secondary barrier. Drug diversion from the skin into the systemic blood circulation is desirable for systemic therapy but hampers local drug delivery and deposition in any peripheral tissue. Alternative and better methods for drug delivery across the skin are therefore needed for systemic and local non-invasive treatment.

3.2 Technology Evolution

A fresh approach to transdermal delivery of drugs started with vesicle application on the skin surface. The initially employed phospholipid vesicles (Mezei and Gulasekharam 1980, 1982; Mezei 1985) and the following vesicles made from nonionic surfactants (Handjani et al. 1989; Hofland et al. 1989, 1994; Schreier and Bouwstra 1994) all contained sterol derivatives (either cholesterol or cholesteryl sulphate, respectively), to stabilise the vesicle's bilayers and to prevent drug leakage or vesicle aggregation. The even more recently introduced vesicles based on skin lipids also included cholesterol sulphate.

Whether they are vesicular or not, all otherwise useful drug carriers (simple lipid vesicles (liposomes), niosomes, micro- or nanoparticles—including solid lipid nanoparticles— and micro- and nano-emulsions) share the problem of nearly complete confinement to the skin surface (Schreier and Bouwstra 1994; Cevc 1997, 2004). The reason is narrowness (<0.4 nm) of the naturally occurring conduits in the stratum corneum, which are too tiny to let any such non-deformable 'carrier' pass (Cevc et al. 2003; Cevc 2004).[1] To transport efficiently a drug through the skin barrier with a carrier, the latter must be able to widen (to around 20–30 nm and at most 40 nm) and/or to trespass (the hydrophilic) pathways in the skin barrier without breaking apart. A drug concentration-independent, sufficiently high, force acting on the carrier and a sufficiently high carrier adaptability and stability,[2] allowing pore entry without the carrier breaking, are the two key prerequisites for such crossing. Any aggregate not fulfilling all these conditions is doomed to fail as a transcutaneous drug carrier.

To date, only a few kinds of drug carriers with the desired characteristics were tested with success on humans, thus confirming the controlled and/or targeted drug delivery across intact skin according to expectation: the specially designed, complex (i.e. at least bi-component), unusually deformable vesicles.[3]

The 1st generation of complex deformable vesicles relied mainly on phospholipid-surfactant blends (Cevc 1992).[4] The synergistic phospholipid-amphipat or drug mixtures were the basis for the 2nd generation of such vesicles, which contained relatively less surfactant and/or more drug molecules (Cevc and Vierl 2003, 2004). The most recent, 3rd generation offers the most advanced and versatile deformable vesicles. Their bilayers are comprised of phospholipid or non-phospholipid amphipats, or amphipat combinations, with the right effective tail cross section, A_c (Cevc 2012a, b). To yield adaptable bilayers that form deformable vesicles, each hydrophobic chain in the bilayer should occupy $\leq 0.43 \pm 0.05$ nm^2. Such value can either be assured intrinsically (by choosing the right bilayer component) or by addition of well-chosen suitable (and often ionic) molecules that have some affinity for bilayer-water interface, which helps them affect amphipat packing within the bilayer. This recognition allows, for example,

[1] Exceptions to the rule, the transepidermal shunts (especially the pilosebaceous units and corneocyte-cluster junctions), are too sparse to be really useful.

[2] Formulations which do not a priori take into account, and compensate for, the drying-induced concentration changes on non-occluded skin are likely to result in vesicle aggregation and fusion on the skin or else in vesicle disintegration or breaking on or in the skin. This probably explains the discrepancy between the results obtained by our group and other researchers in the field.

[3] The first, two-component class of such vesicles was described in 1992 and is known under the trademark Transfersome® of IDEA AG. All the later described related vesicles (e.g. elastic liposomes, ethosomes) contain several bilayer components as well. They should also transport drugs across the skin barrier, arguably relying on a similar mechanism of action. However, to be efficient, the elastic liposomes or ethosomes would have to be stable in addition to being elastic, which is not the case. Most, if not all, of the elastic liposomes described in scientific papers break and/or fuse on an open skin surface. All ethosomes inevitably lose the softening alcohol after a non-occlusive epicutaneous application, either through evaporation or transepidermal diffusion, and then revert into relatively simple bilayer vesicles, liposomes.

[4] The few exceptions designed with a similar application goal in mind consisted of the single-chain nonionic surfactant-cholesterol mixtures, which form flexible bilayers but less stable vesicles than phospholipid-surfactant blends.

preparation of the 3rd-generation vesicles from nonionic surfactants without inclusion of sterols.

Complex deformable vesicles of all three generations can diminish their unidirectional cross section reversibly, influenced by anisotropic stress, relying on the form-dependent components demixing within or near the vesicle-forming bilayer (Fig. 3.2, top). This allows each such vesicle to adjust its shape to a constriction, e.g. a pore in a barrier (Cevc et al. 2003), whether the latter pre-exists in an artificial barrier (Fig. 3.2, middle) or is in the natural skin primary barrier through a narrow pathway widening (Fig. 3.2, bottom). In either case, the vesicle overcomes the constriction after a deformation driven by the vesicle-independent trans-barrier gradient (Cevc 2004).

The gradient-driving trans-barrier motion may have different origins ('gravitation' (Gompper and Kroll 1995), electrostatic potential, hydration (Cevc and Gebauer 2003), etc.) but must act on most, if not all, molecules comprising the carrier to be effective. Presently practically relevant is just the naturally occurring water activity gradient across the stratum corneum. Such gradient cannot only drive but also guide a superficially hydrophilic deformable vesicle through the lowest resistance conduits in the skin primary barrier, through which water molecules normally evaporate in the opposite direction. The boundaries of the involved conduits are the imperfectly adjoined and/or packed hydrophilic surfaces of corneocytes or inter-corneocyte lipid layers in the stratum corneum (Fig. 3.1, right bottom). All this happens without affecting the skin barrier irreversibly or adversely.

A superficially hydrophilic and deformable vesicle can therefore deliver its drug cargo across the skin barrier spontaneously in a well-controllable fashion; the self-optimising vesicle's ability to penetrate a barrier driven by the naturally occurring hydration gradient across the non-occluded stratum corneum is the enabling factor. After the stratum corneum crossing, the vesicle can dispose of its cargo in one of the following ways. (1) It can deposit the drug in the viable skin reached by the vesicle (Cevc and Blume 2004); (2) it can deliver the drug into the skin plus subcutaneous tissue (Cevc and Blume 2001; Cevc et al. 2008a, b); (3) it can release the drug into the

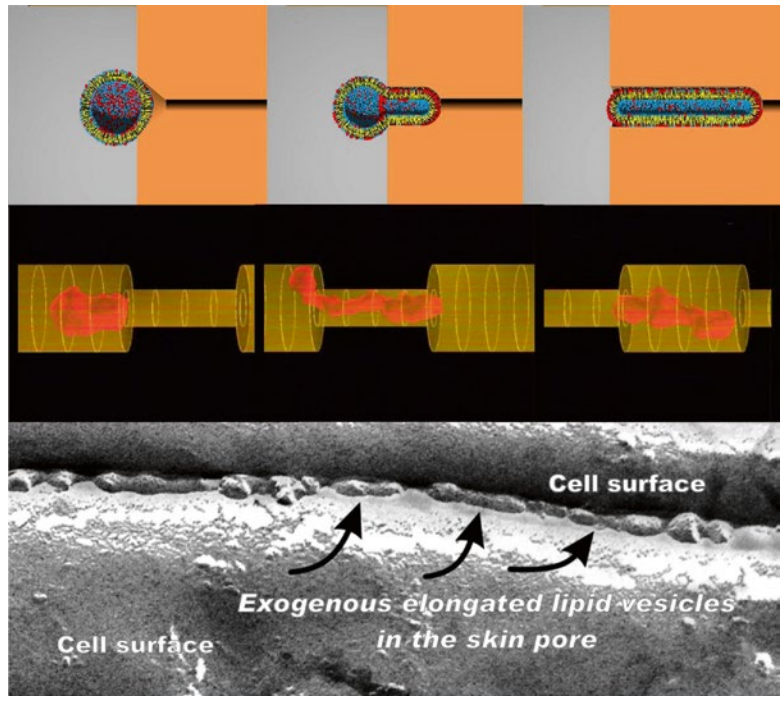


Fig. 3.2 *Top*: computer-simulated distribution of more (*red*, e.g. surfactant) or less (*blue*, e.g. a phospholipid) water-soluble molecules with the hydrophobic (*yellow*) chains arranged in a mixed amphipat bilayer as a function of predefined vesicle shape. *Middle*: a supercomputer (CRAY) simulation of a highly deformable, infinitely permeable, non-destructible vesicle forced by a horizontal gradient ('quasi-gravitation') into a pore with 0.5 smaller diameter. *Bottom*: an electron micrograph of elongated, deformable vesicles in an inter-corneocyte hydrophilic conduit within the human stratum corneum after an application of a lipid preparation on open skin surface (From Cevc and Vierl 2010, with permission)

skin, from where the drug then diffuses into the blood circulation (Cevc et al. 1998; Guo et al 2000; Cevc 2003); and (4) it can carry the vesicle-associated drug further via lymphatic drainage (Paul and Cevc 1995), which the carrier reaches through fenestrations in the dermal lymph capillaries. The carrier penetration depth, and thus the local reach of the vesicle-mediated drug delivery, depends on the vesicles' adaptability and, for properly optimised carriers, on the locally applied drug dose per area (Cevc et al. 2008b).

3.3 Technology Description

The self-optimising deformable vesicles of the first generation designed specifically for transcutaneous delivery of small or large drugs were normally comprised of the bilayer-forming phosphatidylcholine and a biocompatible surfactant, such as sodium cholate, or polysorbates, such as polysorbate 20 or 80 (Cevc 1992, 1997; Cevc et al. 2003; Simões et al. 2004, 2005a, b). The other proposed additives included alkyl- or acyl-polyoxyethylenes, such as Brij 38, 96 and 98 (also known under the trade names Genapol, Thesit or Lubrol) or Myrj 45 and 49, fatty oligoglycerol derivatives and glycolipids, amongst others (Cevc 1992).

The common feature of all molecules added to phosphatidylcholine to increase the resulting bilayer adaptability, and thus vesicle deformability, was their ability to destabilise and potentially solubilise the bilayer. The preferential gathering of the vesicle-destabilising additive at the most curved bilayer sites softened the bilayer locally and thus made the vesicle more adaptable. The top panel of Fig. 3.2 illustrates the phenomenon.

Proper choice and balance of at least two dispersion components with different water solubility are thus key to obtaining deformable *and* stable aggregates (Cevc et al. 2002). Many formulation developers are unaware of, or neglect, the latter need, which can make or break the success of an application in vivo. Partial drying of a dispersion on skin surface, which inevitably changes the amphipat-water ratio and thus the actual carrier composition, magnifies the challenge. In a way, every successful deformable vesicle dispersion contains 'pro-Transfersomes'.

Changing composition is an issue with any kind of dispersion that is applied non-occlusively. Ethosomes, for example, are prepared by adding to a phospholipid ethanol (Godin and Touitou 2003; Dubey et al. 2007), which then binds to the lipid headgroup and thus softens the resulting bilayer (Vierl et al. 1994). Bilayer softening depends on the effective lipid-alcohol ratio and the surrounding water concentration, however. It therefore changes if either of the two formulation characteristics alters, which always happens after a non-occlusive epicutaneous application of ethosomes. An improvement of transcutaneous drug delivery by ethosomes is therefore a consequence of lipid fluidisation and sometimes extraction rather than of the vesicle-mediated delivery. At best, the lipid-dependent skin partial occlusion caused by ethosome vesicle collapse on skin surface supports the well-known skin permeation-enhancing effect of ethanol.

Ensuring the right bilayer composition and sufficient vesicle stability during stress of drying on skin surface is generally difficult, as the outermost skin layer, the stratum corneum, normally contains less water than the applied dispersion. The latter therefore inevitably loses water into the surrounding, except when ambient humidity is close to 100 %.

The reasons for the normal skin surface dryness are the special composition and anatomy of the stratum corneum. This skin part consists of flat and partly overlapping corneocytes organised in stacks and tightened by intercellular lipids (see the dark material between the cells shown in the right top panel of Fig. 3.1). Such cellular organisation and sealing make the skin nearly impermeable to all but rather small lipophilic molecules (with the size-dependent skin permeability) and water (which can cross the organ at a rate of less than 0.5 mg h^{-1} cm^{-2}).

Some cell-cell or cell-lipid contacts are less tight than the others (Schätzlein and Cevc 1998). Such contacts, and/or the sites at which the lipidic seals are imperfect, form tiny (<0.4 nm) hydrophilic conduits across the stratum corneum (Fig. 3.1, lower right). One can widen these

conduits into the hydrophilic passages with ~20–30 nm cross sections by applying unidirectional pressure on them (Cevc et al. 2003) or by exposing them to electro-osmotic stress (Aguilella et al. 1994). A non-occlusive epicutaneous administration of the superficially hydrophilic and highly deformable carriers/vesicles exerts such pressure and triggers the following motional sequence (Cevc and Gebauer 2003). A hydrophilic vesicle threatened by dehydration on skin surface seeks water. The water-seeking vesicle senses humidity within the conduits that let water evaporate from the skin into the ambient. The vesicle enters one of such conduits and moves along the natural hydration gradient within the skin barrier until it reaches the well-hydrated and viable skin part, where water abounds and the gradient ceases. The hydration-dependent vesicle motion therefore stops here, and from then on any vesicle motion relies on the push by the following vesicles, which still sense the gradient, on intercellular fluid (lymph) motion, or on both.

A carrier vesicle is at least two times and typically four to six times larger than an average widened conduit in the stratum corneum. A vesicle can therefore trespass such conduit only if the carrier size, following vesicle deformation in at least one direction, effectively becomes smaller than the widened conduit diameter. High bilayer flexibility and permeability in the conduit are both necessary for this (Cevc et al. 2003). (The relatively high bilayer permeability allows the excess intra-vesicular water to escape from a deformed carrier and the excess drug on the vesicle outside to enter the carrier.) More traditional vesicles (liposomes, niosomes) fail to fulfil one or both of these conditions (Cevc 1996, 1997, 2004; Bouwstra and Honeywell-Nguyen 2002; Honeywell-Nguyen et al. 2002, 2006; Bouwstra et al. 2003).

Drug delivery and biodistribution, starting with an epicutaneous application of deformable vesicles, thus commence in the well-hydrated and viable skin tissue. Small and water-soluble drugs must first leak out or dissociate from the carriers to diffuse further into blood whereupon they can be systemically active. The intercellular fluid flow transports further the larger released drugs or the carriers themselves, which are unable to enter directly cutaneous blood vessels and consequently bypass cutaneous clearance. This can bring the carriers and the associated drugs deep below the application site (Cevc and Blume 2001; Cevc et al. 2008b), unless the carriers are diverted into the fenestrations in dermal lymph capillaries and then reach the systemic blood circulation indirectly (Cevc 1997), potentially ending up in the liver (Cevc 1997).

The first step in the free drug transport (trans-barrier diffusion) leads to the second step (cutaneous clearance into blood). A sufficiently deformable vesicle overcomes the skin barrier without breaking, i.e. as a large entity, and therefore avoids cutaneous clearance. This is most prominent after the saturation of peripheral subcutaneous tissue with carriers, when the applied dose per body weight is high. Before that, an increase in the epicutaneously applied carrier dose mainly influences the maximum depth reached by the carriers.

We have shown in earlier preclinical studies that hydrophilic deformable vesicles can deliver pharmacological agents with different molecular size and lipophilicity (cyclosporin, glucocorticosteroids, lidocaine, tetracaine, tamoxifen, ketoprofen, etc.) efficiently across the mammalian skin barrier. We also delivered with the deformable carriers much larger molecules, such as calcitonin; insulin; interferon-α and interferon-γ (Cevc 1997, 2004); Cu-Zn superoxide dismutase (Simões et al. 2009), all in biologically active form; we likewise transported across skin barrier serum albumin (Paul and Cevc 1995) and gap junction protein (Paul et al. 1995) in immunologically active form. We designed formulations for all these studies so as to deliver drug molecules mainly to the site of their desired action: the skin or the systemic blood circulation. We achieved a high local drug concentration with cyclosporin and glucocorticosteroids included into the slow-release formulations, for example (Cevc and Blume 2003, 2004). Conversely, we brought different kinds of the tested polypeptides into systemic blood

flow using fast-release formulations (Cevc et al. 1998; Cevc 2003).

We moreover succeeded in targeting nonsteroidal anti-inflammatory agents into peripheral and deep subcutaneous (muscle and joint) tissue. The key to success was the use of such vesicle compositions that ensure close to 100 % drug loading on skin surface (after around 40 % in the original dispersion, see Fig. 3.3, left) whilst assuring to the vesicles' maximum possible adaptability (see Fig. 3.3, right) plus sufficient stability (data not shown) after an epicutaneous application. Preclinical tests with the resulting formulation on pigs revealed over 75 % drug bioavailability (Cevc et al. 2008b). The tests also confirmed that drug persisted in the deep subcutaneous tissues below an application site longer if it was delivered with the vesicles (see Cevc et al. 2008b and Fig. 3.8). Our preclinical study results are thus entirely consistent with the positive therapeutic results from the phase I (Fesq et al. 2003; Rother et al. 2009) to phase III clinical studies (Rother et al. 2007; Kneer et al. 2009; Conaghan et al. 2013). The latter involved an optimised nonsteroidal anti-inflammatory drug (NSAID) formulation, IDEA-033 or Diractin® (IDEA AG, Germany).

More recent studies done with the NSAID loaded and the corresponding drug-free vesicles, comprised of soybean phosphatidylcholine plus some polysorbate 80 (Cevc et al. 2008a), suggest that the therapeutic effect of either vesicle kind can be comparable in humans (Conaghan et al. 2013), if the treated inflammation and pain are not too severe. In the German market, one can buy since 2012 a drug-free commercial product (Flexiseq®, ProBonoBio, Germany), which according to its distributors contains deformable vesicles and relates to the data published by Conaghan et al. (2013). A recent publication of the results obtained with several formulations containing the 3rd-generation deformable vesicles implies superiority of such vesicles (Cevc 2012c) over the previously tested drug-free vesicles (Conaghan et al. 2013), which become stiffer after NSAID elimination (Cevc et al. 2008a) and then resemble traditional liposomes tested by Cevc (2012c).

3.4 Vesicle-Skin Interactions

3.4.1 Aggregate Effects on Mammalian Skin Structure and Properties

Any dispersion applied on the skin surface changes skin properties at least transiently. If it does nothing else, a water-based dispersion temporarily increases skin hydration, which is known to lower the skin barrier to transport (Warner and Lilly 1994; Warner et al. 1999, 2003; Schaefer et al. 2002).[5] An early observation was that pretreating the skin with liposomes is useless in comparison with the drug-loaded liposomes application (du Plessis and Müller 1994). This is consistent with our conclusion and also holds true for the functional skin penetrants (e.g. deformable vesicles), albeit for a different reason. We therefore conclude that any vesicle must be present on/in skin *during* drug transport to be in any way effective. A deformable drug-loaded vesicle can then carry its drug payload through the skin barrier, whereas a non-deformable vesicle improves drug diffusion by increasing skin hydration. In the latter situation, the vesicle-associated drug remains on the skin surface, however.

Organic solvents or harsh surfactants from a dispersion can fluidise and/or extract intercellular skin lipids (which happens with ethosomes or sodium dodecyl sulphate preparations, for example). The extraction probability is higher when the dispersion accommodates skin lipids especially well (which is the case with the skin lipid dispersions used as 'carriers', e.g owing to the principle that 'like likes like'). Phospholipids used in traditional liposomes or in the related 1st- and 2nd-generation Transfersome® vesicles do not extract skin lipids in appreciable quantity, as neither phospholipids nor the surfactants, which must be included to form such vesicles, match skin lipids well.

[5] Water disrupts the stratum corneum similarly to surfactants, but with a slower onset. After 2 h, intercellular lamellar bilayers alter, but do not yet roll up. This happens after 6 h, however. After 24 h of exposure to water, corneocytes in the skin primary barrier dissociate more extensively (Warner et al. 1999).

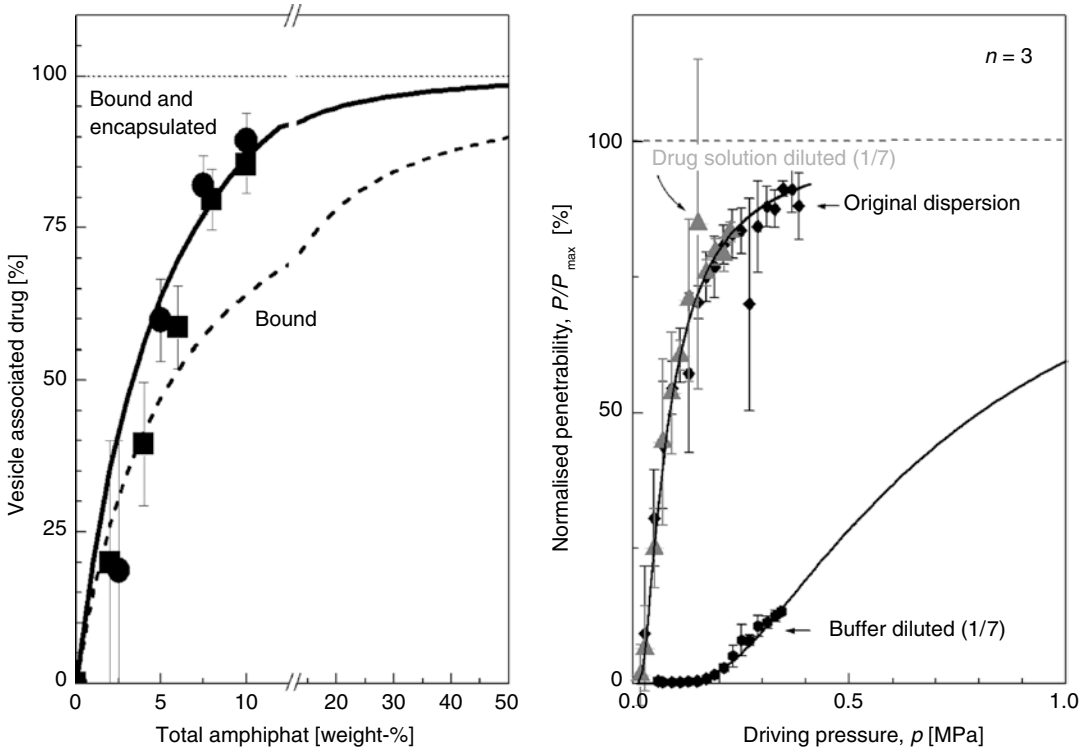


Fig. 3.3 *Left*: drug (NSAID) association with the mixed lipid vesicles comprising of phosphatidylcholine and polysorbate 80 as a function of total amphipat concentration, which approaches total carrier weight with increasing concentration. The curves were calculated with the appropriate drug association model and reveal around 20 % encapsulation and some 50 % binding to vesicle bilayer in the original, 10 weight-% dispersion. In the up-concentrated dispersion on skin surface (~50 weight-%), practically 100 % of drug molecules are vesicle associated. *Right*: penetrability, normalised to similar dispersion viscosity, of a nanoporous filter barrier ($r_v/r_{pore}>4/1$) to different suspensions containing vesicle aggregates with a high ('original dispersion', 'drug solution diluted') and low ('buffer diluted') adaptability

We were the first to show clearly (Cevc 1996; Schätzlein and Cevc 1998) that the traditional, normally rigid, liposomes and micelles cannot penetrate the skin primary barrier, thus rebuking earlier claims to the contrary (Mezei 1985). By analysing their confocal scanning laser micrographs, van Kuijk-Meuwissen et al. (1998) likewise found that the label applied non-occlusively in micelles and traditional gel-state liposomes does not penetrate as deep into skin as the label applied in the traditional liquid-state vesicles. The authors thus measured the highest fluorescence intensity in the dermis of the skin treated with the fluid-state, 'flexible bilayer' vesicles. To add further detail, van den Bergh et al. (1999) used two-photon fluorescence microscopy and observed distinctly different penetration pathways after a non-occlusive application of the elastic (single-chain) or rigid (cholesterol-stabilised) vesicles. The former created thread-like channels within the entire stratum corneum and abolished the polygonal corneocyte cell shape. In contrast, the fluorescently labelled rigid vesicles were confined to the intercellular spaces of the upper 2–5 μm of the stratum corneum, which also retained its structure. We made similar observations with the confocal scanning microscopy and moreover identified an additional, different kind of the hydrophilic penetration pathways, i.e. conduits, through the skin primary barrier (Schätzlein and Cevc 1998; Cevc et al. 2003). Very recently, a group from Denmark (Brewer et al. 2013) used spatially resolved two-colour diffusion measurements to study fate of various epicutaneously applied vesicles. Unfortunately, the carefully conducted study was done in vitro (and with the

preparations in which the extravesicular dye had been removed prior to dispersion application). This limits validity of the study conclusion 'that the penetration of intact liposomes (including the 1st generation Transfersome® vesicles) is highly compromised by the skin barrier' only to the specific investigated test system.

Use of single-chain surfactant vesicles with a tendency to aggregate or disintegrate on/in skin is one possible reason for finding only few elastic vesicles in the viable epidermis (Honeywell-Nguyen et al. 2002, 2006). An unrelated reason is reliance on in vitro tests (Brewer et al. 2013). In such tests, even deformable vesicles can penetrate just to the end of hydration gradient in the stratum corneum. Lack of intercellular fluid motion in the underlying epidermis ex vivo creates an unstirred layer there, which blocks vesicles' further transport into the viable epidermis. Instead of finding a quasi-continuous vesicle distribution profile in the skin (Cevc et al. 2003), one finds a decaying profile in the stratum corneum terminated by a peak near the basal layer, i.e. at the epidermis-dermis junction. Vesicle penetration tests with the mammalian skin in vitro, for related reasons, also suggest vesicle size dependence (Verma et al. 2003) that we never observed with deformable vesicles in vivo.

The fate of vesicles that overcome the skin primary barrier resembles that of intradermally injected vesicles.

3.4.2 The Skin (Immune) Response to Epicutaneously Applied Antigens and Aggregates

The skin is the largest mammalian immune organ and the primary site of the preferential/selective reaction to an epicutaneously applied antigen or to an invading pathogen. The numerously predominant keratinocytes can secrete cytokines,[6] which then interact with dendritic cells (the Langerhans' cells ('LC') and the dermal dendritic cells ('dDC')) to keep immunoreactivity and tolerance in balance. Secretion of cytokines IL-1 and GM-CSF by keratinocytes, as bystanders, thus enhances antigen presentation by T cells (Sugita et al 2006), and cytokine TGF-β1 secretion induces migration of LCs and increases dDC number in the draining lymph nodes, which results in inflammation (Mohammed et al. 2013). In turn, antigen presentation by keratinocytes promotes tolerance (Bal et al. 1990). Despite the residual uncertainty about the interdependent regulation of epidermal cell activity, it is already clear that the epidermal cells can trigger maturation of the Langerhans' cells in the epidermis and of dendritic cells in the dermis. This leads to their migration into the draining regional lymph nodes and orchestration of the resulting immune response (Randolph et al. 2005; Udey 2012). Reportedly, nanoparticles can serve as vectors for antigen targeting to the lymph node-residing dendritic cells via interstitial flow and can also activate these cells by in situ complement activation. Reddy et al. (2007) have shown that interstitial flow-mediated transport of intradermally injected, ultra-small nanoparticles (25 nm) efficiently into lymphatic capillaries and their draining lymph nodes, targeting half of the lymph node-residing dendritic cells; 100 nm nanoparticles were only 10 % as efficient. It is thus reasonable to expect that antigen-loaded deformable vesicles, applied non-invasively, that also enter lymphatic capillaries via fenestrations, can be effective too. Possibly/probably the latter are subject to a different size limitation, however, if any. We, indeed, measured the draining lymph node mass to increase after such vesicle application on skin surface, which supports the expectation.

We have already shown that repeated applications of different macromolecular antigens (serum albumin (Paul et al. 1995), gap junction protein (Paul and Cevc 1995), tetanus toxoid (Chopra and Cevc 2013)) can elicit a strong, systemic immune response. Depending on the used antigen nature, the response can be quantitatively similar (Paul et al. 1995; Paul and Cevc 1995) or else attain a bias toward Th2 (i.e. 'T helper') cell stimulation (Chopra and Cevc 2013), compared with the animals'

[6] The most prominent examples are interleukins (IL), such as IL-1, IL-1β, granulocyte macrophage colony-stimulating factor (GM-CSF), transforming growth factor (TGF-β1) and tumour necrosis factor (TNF-α).

immune response to injections of similar immuno-vesicles. Encouragingly, entirely non-invasive immunisation with the deformable vesicles used as tetanus toxoid vehicles completely protected the test animals against an otherwise lethal challenge with 50 times the lethal tetanus toxin dose. Skin irritation or the organ barrier disruption played no role in this desirable prophylactic effect, as independent tests confirmed lack of inflammatory cytokines secretion and preservation of normal skin structure in the tests done with skin surrogate and the deformable vesicles in vitro (Chopra and Cevc 2013). In contrast, simple 'immuno-vesicles' (antigen-loaded liposomes) did not trigger any significant immune response after epicutaneous applications. At best, such vesicles can serve as a superficial reservoir of immnostimulating small molecules/skin irritants and/or irritate the skin mildly by causing a transient (quasi)occlusion. The epicutaneously applied mixed micelles are also not effective as antigen carriers (Paul et al. 1995).

All other reports on induction of the cutaneous immune response refer to a more or less complete and radical disruption of the skin barrier (Mikszta and Laurent 2008), triggering a massive secretion of cytokines (e.g. by keratinocytes) and initiating a cascade of events leading to a strong local immune response. (The flip side is that such over-irritation masks any initial events associated with antigen entry into skin.) Applications of an antigen plus a bio-adjuvant (especially cholera toxin) onto abraded skin can correspondingly induce a significant immune response, but is only weakly protective (Glenn et al. 1998).

Well-designed deformable vesicles can conversely demonstrably deliver antigens in highly active form into an essentially unperturbed skin. This offers unprecedented means for detailed studies of cutaneous immuno-stimulation and activation in vivo, including but not limited to exploration of cutaneous immune response to a locally (and non-invasively) applied antigen as a function of the antigen characteristics, without any carrier- or delivery method-dependent bias.

3.5 Research and Development

Deformable Vesicle Preparation Any kind of deformable vesicles can be prepared relying on the published and patented procedures (Cevc 1992, Cevc 2012a, b, c; Cevc and Vierl 2003, 2004). Briefly, to prepare the first-generation deformable vesicles, one blends phosphatidylcholine (e.g., from soybean = SPC) in ethanol with appropriate quantity (see the next paragraph) of a biocompatible surfactant. If desired, one can then remove ethanol. Suitable buffer addition to the resulting blend always generates a coarse dispersion of vesicles (typically with the total lipid concentration $\leq$15 wt-%). The dispersion homogenisation (e.g. with ultrasound, a high-velocity homogeniser or a filtration through adequately narrow pores) narrows down the vesicle size distribution width and ensures vesicle unilamellarity. If desired, one can subsequently freeze-thaw the dispersion two to three times to catalyse vesicle fusion and thus gain larger vesicles. Thereafter, one can repeat homogenisation, ultrasonication or some other pharmaceutically acceptable mechanical fragmentation to restore the preferred vesicle size. The latter, as determined with the dynamic or static light scattering (Elsayed and Cevc 2011a), should preferably be around 100 nm, but is not application deciding (owing to the negligibly small transport sensitivity to the average vesicle diameter). One should *not* separate the non-associated/non-encapsulated drug from vesicles in dispersion, as the majority of the originally non-associated drugs—in a well-designed preparation—will associate with the vesicles after their partial drying on/in the stratum corneum. The non-associated drug will moreover be left on the skin surface acting as a barrier.

It is crucial to realise that there is no general 'Transfersome® recipe': one must adjust the vesicles and their suspending medium composition individually to each drug and the specific payload. The high bilayer adaptability and vesicle stability are the two most crucial selection criteria in the process. Empty vesicles prepared from 8.7 wt-% SPC, 1.3 wt-% sodium cholate or 10 wt-% SPC/polysorbate 80 mixture with 4/1–3/1 mol/mol ratio in a pH ~ 7.5 phosphate buffer (10–

25 mM plus up to 8.5 vol-% ethanol) can cross the mammalian skin barrier well, for example. Buffer acidification to pH 6 diminishes deformability of the former, but not of the latter kind of vesicles, and incorporation of ≤5 wt-% of an NSAID (such as diclofenac) destroys the resulting vesicle stability, owing to the surfactant-like activity of most NSAIDs. Many other drugs pose similar formulation challenges.

Deformable Vesicle Characterisation One can gauge the relative ability of certain carriers to penetrate an artificial nanoporous barrier as a proxy to the similar carrier passage through a skin with the already widened hydrophilic conduits (Wachter et al. 2008). To conduct the test, one takes a membrane filter with the fixed pores number density (the higher the better) and pore size (the most relevant: 20–30 nm). By applying different hydrostatic pressures, p, on the filter, one drives the tested vesicle dispersion through pores in the filter with the pressure-dependent flux density, $j(p) = \Phi(p)/\text{area}$, or transport rate; either is diagnostic of the barrier penetrability, $P(p)$, to the tested vesicles (cf. Fig. 3.4). One can describe the measured data set with the following formula, based on activation energy concept (Cevc et al. 2003):

$$P(p) = P_{\max}\left[1 - \operatorname{erf}\left(p^*/p\right)^{0.5} + \left(4p^*/\pi p\right)^{0.5}\exp\left(-p^*/p\right)\right]$$

erf is error function. $P_{\max}$ is the directly measurable (e.g. by using a very high flux-driving pressure or by using the solubilised vesicles, i.e. the mixed micelles unconfined by pores in the barrier) maximum barrier penetrability (and thus

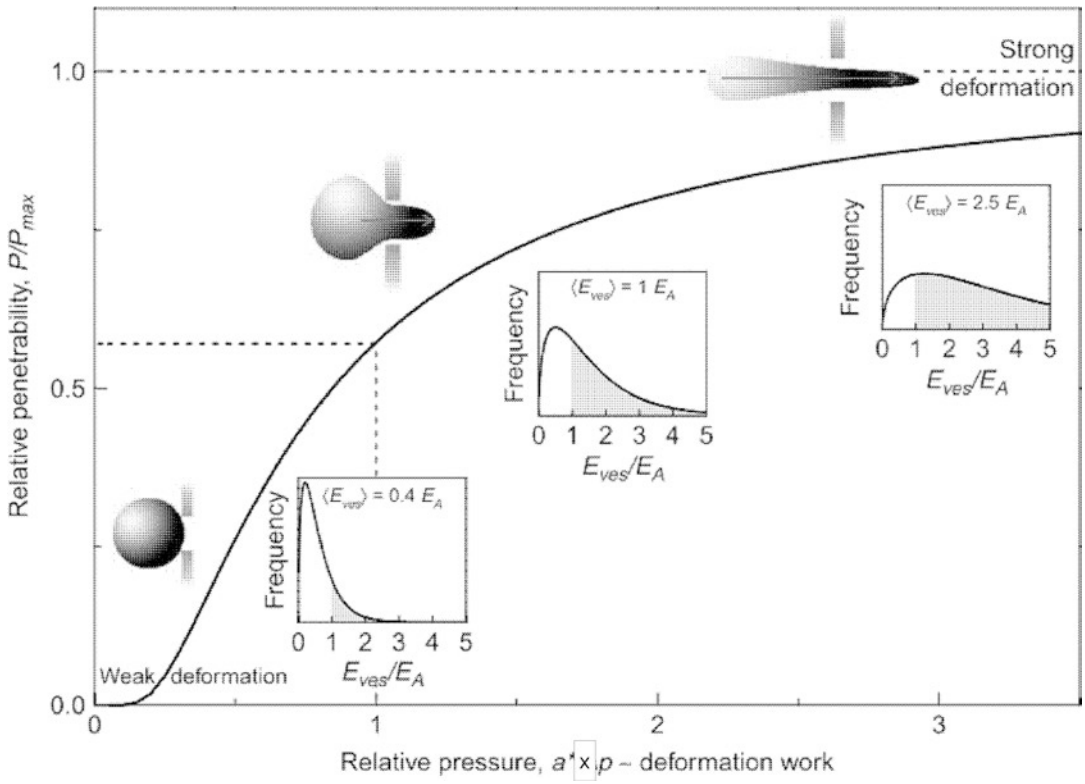


Fig. 3.4 Illustration of relative pore penetrability to a vesicle, $P/P_{\max}$, as a function of the driving pressure difference across the pore, Δp, and of the vesicle energy, E_{ves}, based on the Maxwell-Boltzmann distribution. The latter is illustrated for three of the intermediate states in insets (From Wachter et al. 2008)

depends on filter porosity). The 'flow-activating pressure', p^*, is the only adjustable model parameter in the above equation. It describes the tested system resistance to the trans-barrier transport in pressure terms. $1/p^*$ thus defines the characteristic penetrability of a narrow, constant size, pore, by the carrier, in inverse pressure units. It thus reflects, indirectly, the penetrating carrier's adaptability, which is composition and driving pressure dependent (see Fig. 3.5, left panel). The latter dependency reflects consequences of the local bilayer composition adjustment to the enforced vesicle deformation. Along with the test, one can also check the carrier's size stability by measuring the average carrier size before and after the filter passage with the dynamic or static light scattering, after appropriate flow-dependency correction (Cevc et al. 1998; Wachter et al. 2008).

In an alternative, instrumentally simpler, test, one measures the time dependence of vesicles size diminution caused by an external stress (Cevc 2012a). To generate the latter, one can use a power and duty cycle-controlled ultra-sonifier with a titanium tip or horn transducer. Alternatively, one can use a frequency-controlled laboratory homogeniser (e.g. an Ultra-Turrax®, IKA, Germany). The time needed to reach certain final vesicle size (or size diminution), t_{ves}, is proportional to the tested bilayer vesicle adaptability:

$$\kappa_c / \mathrm{RT} \propto p^* = \left(t_{ves}\, p^*_{ref}\right) / \left(t_{ves,\,ref}\,\mathrm{MPa}\right).$$

The result is expressible either in terms of bilayers bending (elasticity) modulus, κ_c, or in the units of p^* (measured in MPa) both after normalisation with respect to the reference vesicle result corresponding to an independently known $\kappa_{c,ref} \propto p^*_{ref}$ value. The right panel of Fig. 3.5 shows some claim-supporting data.

In vivo studies of deformable vesicle migration across the skin primary barrier typically rely on tissue biopsy punches, for deep tissues, or on the vacuum-pulled skin blisters, for cutaneous investigations. To get practically relevant results,

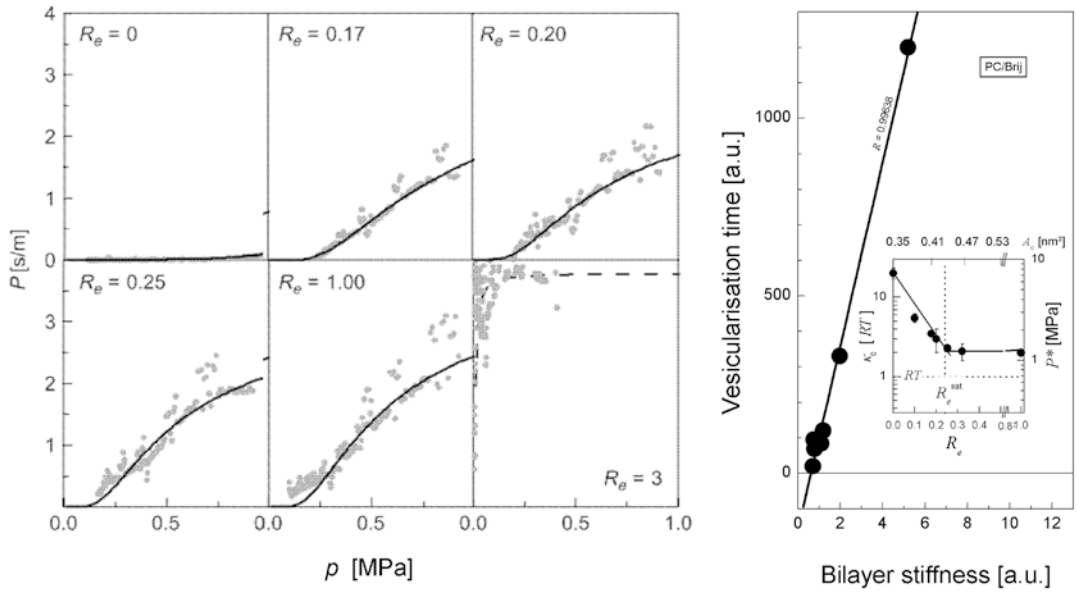


Fig. 3.5 Pore penetration ability (penetrability, P) as a function of trans-barrier driving pressure, p, for simple phosphatidylcholine vesicle suspension (*top left*: $R_e = 0$, $r_{ves}/r_{pore} > 5$; P function: —; P experimental data: grey dots) or for the suspensions of mixed bilayer vesicles with different molar surfactant to lipid ratios R_e. The corresponding mixed micelles' result is shown for comparison at the *bottom right* ($R_e = 3$, $r_{mic}/r_{pore} < 0.7$) (*Left*: Modified from Wachter et al. 2008, with permission. *Right*: From Cevc 2012a, with permission)

one must take such tissue samples from a living subject or animal, unless one can sample the latter immediately post mortem. In vitro experiments with deformable vesicles always yield misleading, false-positive or false-negative, results. For example, even a poor carrier containing its membrane destabiliser, such as a surfactant or an alcohol, can increase skin permeability in vitro by either extracting or fluidising skin lipids (see previous text for more detail). On the other hand, even an excellent carrier, designed to deliver drugs deep below a local application site, can lower transcutaneous diffusion of a drug with an affinity for the vesicle bilayer. The reason is that the bilayer-associated drug molecules are quasi-bound and therefore do not contribute to trans-barrier concentration difference; they are consequently confined to the skin surface as long as the vesicles, to which they are bound, stay there (Fig.3.6).

In vivo-in vitro comparison can resolve the dilemma. If the results of such a comparison are similar, one deals with diffusive transport; if the results are different, the observed transport is arguably carrier mediated (see Fig. 3.7).

When studying drug delivery into skin or some other superficial tissue, one should eliminate, before skin sampling, the residual lipid dispersion from skin surface with a dry or water-soaked cotton swab; otherwise, the superficial tissue values will be erroneously high. One should not use for the purpose an organic solvent, such as ethanol, as the latter is likely to facilitate molecular diffusion from the skin surface into the organ depth. More voluminous peripheral tissue samples, such as the subcutaneous fatty or muscle layers, should be sliced into conveniently thin, individual specimen to determine the studied vesicles or drug penetration profile. Labelled carriers or drugs as well as spectroscopic or chromatographic methods for the extracted drug analyses are helpful.

In an early experiment, we had labelled the first-generation deformable vesicles with ^{3}H-phosphatidylcholine and applied the resulting dispersion on intact murine skin (with shortened

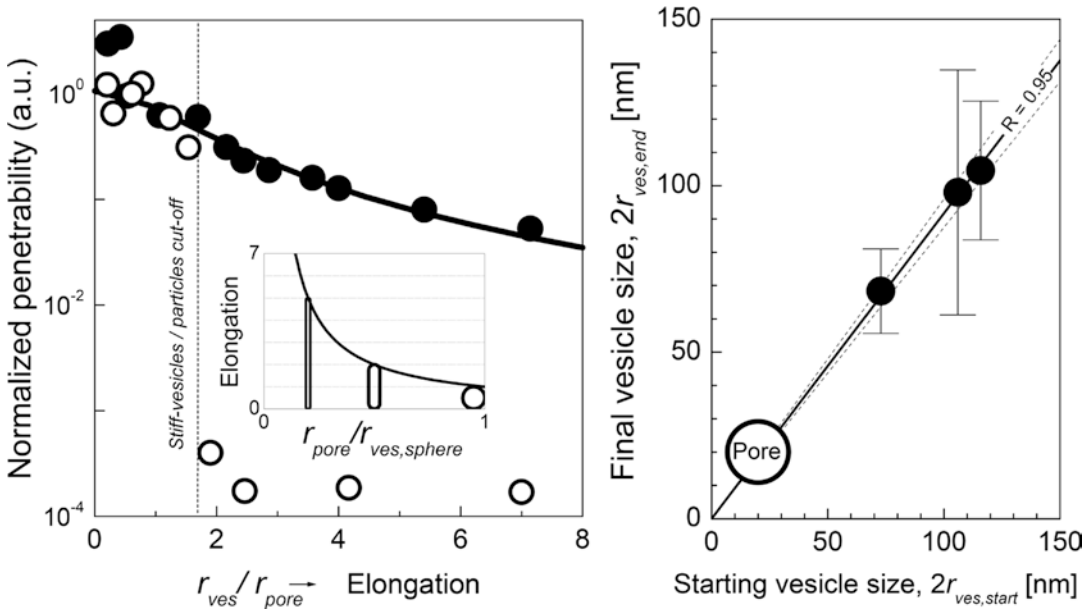


Fig. 3.6 *Left*: penetrability of a nanoporous, semipermeable barrier to a dispersion of deformable vesicles (*full symbols*) or liposomes (*circles*) as a function of relative aggregate/pore size ratio. The inset illustrates the vesicle elongation as a function of this ratio (From Cevc et al. 2003, with permission). *Right*: the final vesicle size after pore crossing compared with the starting vesicle size (From Cevc et al. 2008a, with permission)

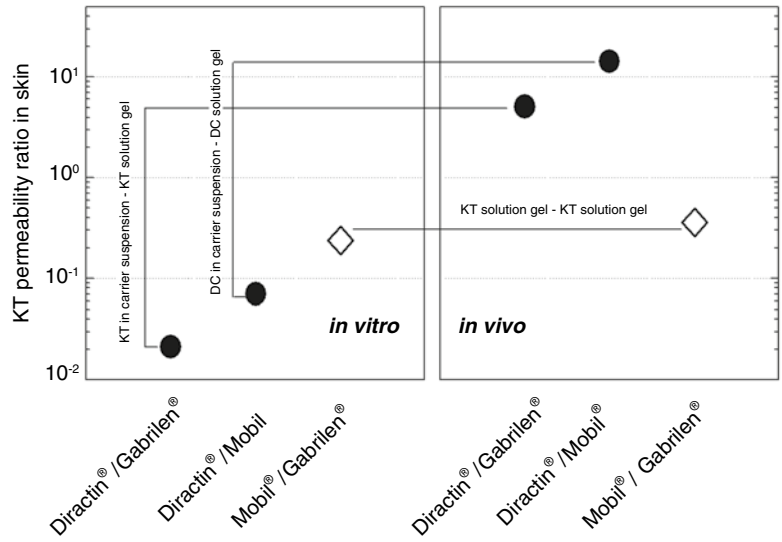


Fig. 3.7 Relative permeability of ketoprofen (KT) in human split-thickness skin in vitro (at $t = 24$ h) and in porcine skin in vivo (at $t = 1$ h), as determined by comparison with the corresponding drug concentrations in the superficial subcutaneous muscles measured with the same preparations in vivo (From Cevc et al. 2008a, with permission)

hair) or else injected the same test dispersion in similar quantity subcutaneously. We then measured the carrier-derived radioactivity as a function of time in the blood, dermis, three different muscles, kidney, liver, spleen and several other organs. After one day, we measured essentially the same radioactivity biodistribution for the injected and epicutaneously applied vesicular dispersions. The vesicle-derived label was measurable in blood 1 h after the test formulation application. Its concentration increased with time and reached a plateau some 6–10 h after the application.

To study intra- and transcutaneous drug delivery mediated by deformable vesicles in mammals, we carried out in vivo experiments with ^{3}H- or ^{14}C-labelled drugs in mice. This revealed that the low-molecular-weight lipophilic drugs, such as testosterone (MW = 288) or triamcinolone acetonide (MW = 434.5), delivered with the vesicles appear in the blood at times that are comparable to those measured for the carrier-attached label (Cevc 1997) but appreciably later than the intradermally injected drug (see, e.g., Cevc et al. 1997); this suggests carrier-mediated transcutaneous delivery of such drugs. The highly water-soluble calcitonin (MW = 3,432), heparin (MW = 7,500) or dextran (MW = 70,000) applied on skin surface in deformable vesicles also appeared in murine systemic circulation with a 4–6 h delay (Cevc 1997), as did the label from the independently tested, comparable, vesicles. The deformable vesicles carried these substances and serum albumin (MW = 64,000; Cevc 1997) through the skin barrier equally well. This suggests lack of molecular size effect on the vesicle-mediated drug delivery through the mammalian skin, at least within practically relevant limits. We even recovered serum albumin in the murine blood with a similar distribution profile as testosterone, based on comparison of ^{125}J- and ^{3}H-radioactivity measurements. The more recent direct measurements of an NSAID drug delivered across porcine skin with deformable vesicles (Cevc et al. 2008b) are consistent with these findings.

Several groups tested insulin delivery with the mixed phosphatidylcholine-bile salt carriers in biological experiments (Cevc et al. 1998; Guo et al. 2000; Cevc 2003). All the published data confirm feasibility of non-invasive transcutaneous transport of this biologically active polypeptide by the 1st-generation carriers (which do not need much fine-tuning when delivering a relatively small quantity of a relatively small peptide). Cevc (2003) highlights some of our own data collected in the early human experiments with a regular, commercial, recombinant human insulin (Actrapid®, Novo-Nordisk, Denmark) associated with a preparation of 90–110 nm large

vesicles. In the corresponding preclinical experiments, we associated carriers with the ^{125}I-labelled insulin and applied the resulting dispersion on murine skin. We subsequently measured the carrier-derived insulin radioactivity distribution in the murine body autoradiographically (Cevc et al. 1998). We detected the highest concentration of the insulin-derived radioactivity in urine, followed by gastrointestinal tract, other soft tissues, and blood. We observed no major difference between the biodistributions resulting from epicutaneous and subcutaneous applications, except for the different lag times, which were 4–6 h longer for the epicutaneous as compared with the subcutaneous group.

In humans, measured and reported 2–30 % lowering of blood glucose level, starting approximately 2 h after an epicutaneous insulin application with the fast-release deformable vesicle carriers (Transfersulin (Cevc et al. 1998; Cevc 2003)). In contrast, the analogously prepared and used insulin in the related mixed lipid micelles or liposomes dispersion did not change the measured blood glucose significantly. It is unclear to date whether this non-invasive kind of insulin delivery will ever be used in clinical practice, but it is clear that the underlying technology is viable.

Studies of slow-release deformable vesicles loaded with an NSAID, ketoprofen, referred to in the publications as IDEA-033 or Diractin®, in humans gave different results. We have measured only low systemic drug concentrations after an epicutaneous administration of such vesicles (ten times lower than after an oral uptake of a similar drug quantity). In turn, we were able to detect much higher local drug concentrations in deep tissues below an epicutaneous application site. We moreover showed in experiments with pigs that these concentrations depend only little on depth below the skin—as one would expect for the non-diffusive, carrier-mediated transport driven only near the involved skin surface.[7] We furthermore observed that the drug concentration, which results from delivery with deformable vesicles but not that based on diffusion-based drug delivery, is controllable by changing the locally applied vesicles, and thus drug, amount per area (see Figs. 3.8 and 3.9), which vindicates the conclusion.[8]

3.6 Regulatory Aspects

More than a hundred products based on traditional transdermal drug delivery approach can be bought, but only a few were introduced recently. This reflects more technological than regulatory limitations, however. Some of the limitations could be resolved by using the new deformable vesicle technology.

Several products based on deformable vesicles have been scrutinised by various national and international regulatory bodies, according to the press releases of IDEA AG. A pharmaceutical product comprised of ketoprofen associated with deformable vesicles (IDEA-033 or Diractin®, IDEA AG, Germany) received marketing approval from the Swiss regulatory agency for treating signs and symptoms associated with osteoarthritis of the knee, but was not launched by the originator company to date for commercial reasons. A different, drug-free, product from the same originator is now commercially available in Germany as a registered medical device, sold by a third party for a similar indication under the trade name Flexiseq® (ProBonoBio, UK).

The fact that several regulators have afforded marketing authorisation to different therapeutic products based on the complex vesicles comprised of phosphatidylcholine and surfactant(s) suggests that other more or less related products could gain marketing authorisation as well. The proviso is exclusive use of pharmaceutically established excipients, which is implemented in all three generations of deformable vesicles. This

[7] Consistent with the conclusion, we observed steep drug concentration decline near the skin surface and only little further depth dependence in earlier experiments done with deformable vesicles loaded with a different NSAID, diclofenac (Cevc and Blume 2001).

[8] Increasing the locally applied dose per area increases drug concentration in deep subcutaneous tissue and vice versa.

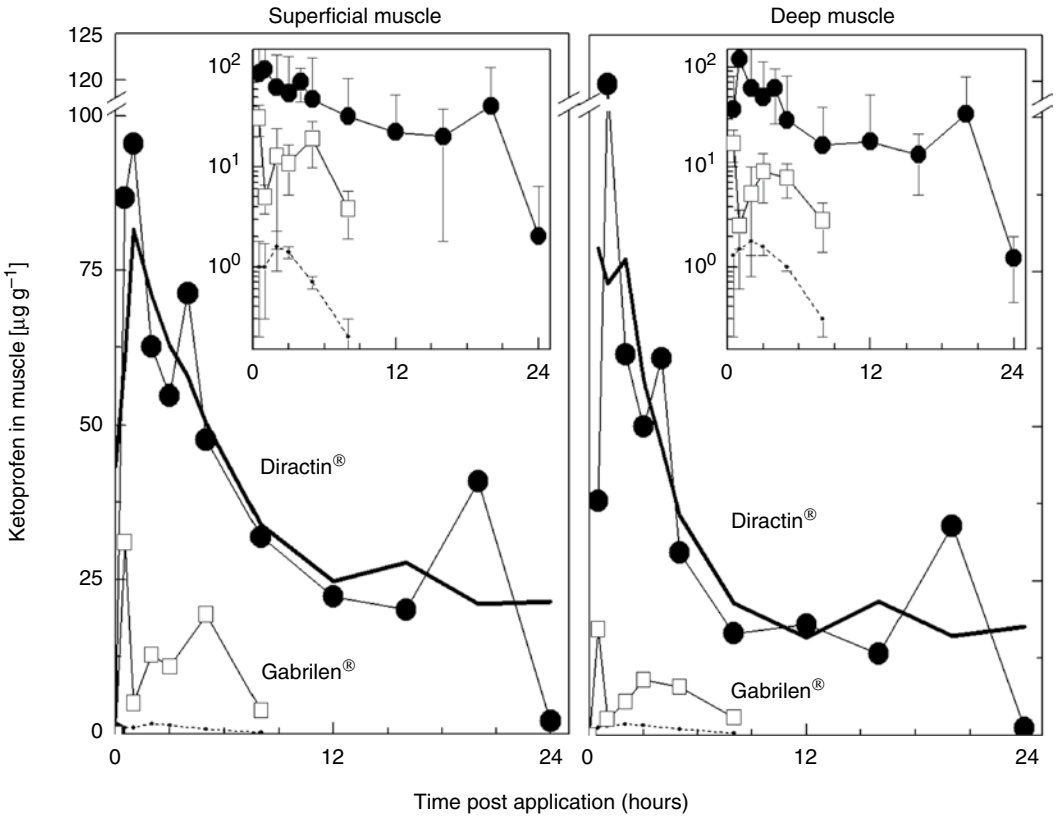
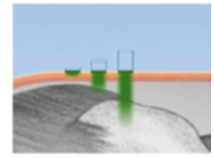


Fig. 3.8 *Left*: temporal dependency of ketoprofen concentration in superficial porcine muscle tissue (0–15 mm below a treatment site) after an epicutaneous application of 50 mg drug in Diractin® (*bullet*, $n = 4$–24, IDEA AG, Germany) or Gabrilen® (*box*, $n = 6$, Kreussler Pharma, Germany). *Lines* should only guide the eye: the thick ones in the main panels give the result of three nearest neighbours averaging for Diractin®. The *thin lines* connect the original data for Gabrilen® or oral ketoprofen (KT). The *small bullets* with the *dashed lines* provide comparison with the results of an oral drug application ($n = 3$). *Right*: The related results measured in deep muscle tissue (15–30 mm below application site). *Insets* show the same data in a logarithmic plot and include standard deviations of the mean (From Cevc et al. 2008b, with permission)

AUC of the area-dose dependent recovery of ketoprofen (KT) in pigs muscles

	Ketoprofen Dose		Muscle	
	Per area *mg cm⁻²*	Total *mg*	Superficial *µg h g⁻¹*	Deep *µg h g⁻¹*
0-8 hours (n = 4)				
KT in vesicles	0.17	17	147	97
	0.50*	**50**	**278**	**266**
	1.50	50	440	429
KT in gel	0.17	17	47	54
	0.50*	**50**	**104**	**44**



*Maximum reasonable dose per knee and a single application
AUC = Area under the curve.

Fig. 3.9 The carrier-mediated, dose-controlled drug targeting into deep subcutaneous tissues using the composite deformable vesicles. Ketoprofen from epicutaneously applied deformable vesicles (IDEA-033 = Diractin®, IDEA AG) but not from a conventional, commercial gel (Gabrilen® gel, Kreussler Pharma GmbH) reaches deep local tissue reliably. The maximum depth of ketoprofen delivery for the former depends on the applied carrier, and thus drug dose, per area. This is not the case for the free drug, diffusing from a commercial gel

fact notwithstanding, any new product involving deformable drug carriers will have to pass certain clinical tests confirming its therapeutic activity and toxicological acceptability.

The principle of deformable vesicle action and the resulting non-invasive delivery effects on the carried drug pharmacokinetics and biodistribution are well understood. The solid theoretical framework underlying deformable vesicle technology moreover allows outcome-optimised study design. This implies relatively low probability of biological tests failure, so long as drug properties and pharmacokinetics are well understood too.

3.7 Position and Advantages of the Technology

People have long considered intact skin as an interesting but challenging site of entry into a body. The underlying, biologically optimised, skin primary barrier is difficult to overcome, indeed, and practically refractory to all but small lipophilic molecules. Properly designed deformable vesicles conquer this barrier without damage and thus overcome the previous limitation on non-invasive intra- and transcutaneous drug delivery. In different words, such deformable vesicles can deliver drugs into skin, into peripheral tissue or into blood and the rest of a body. In contrast, the non-optimised formulations that contain no or too stiff vesicles, including simple hydrogels/ointments/creams and liposomes/niosomes, offer no real benefit for the controlled drug delivery into and across the skin barrier. Addition of chemical skin permeation enhancers can improve the situation in the first but not in the second of these two aspects (and for small drugs only). Skin poration with electrical pulses, ultrasound, thermal injury or various ballistic methods (e.g. powder-jection, high-velocity micro-droplet impact) suffers from long skin recovery period, brings the danger of body infections through the resulting lesions, and does not permit drug delivery in a large quantity. The same is true for the mini-pumps that require skin wound through which such a pump continuously infuses drug molecules into the body.

Deformable vesicles of all three generations therefore stand alone in terms of versatility and effect, as well as gentleness to the skin. They can carry agents of various size, structure, molecular weight, and polarity either through the skin primary barrier in the stratum corneum or beyond the secondary, dissipative barrier of the cutaneous blood plexus. One can manufacture deformable vesicles with the generally available manufacturing tools from the freely available, pharmaceutically acceptable ingredients. One must choose and combine the latter carefully, however, to ensure proper functioning of the resulting vesicles.

Deformable vesicular carriers cannot be characterised adequately using the conventional analytical techniques only, which serve well just for the less innovative drug carriers, such as liposomes. The former kind of carriers, on the one hand, needs extra tests to assess bilayer vesicle deformability. On the other hand, it does not require separation of the free and the carrier-associated drug, as skin barrier anyhow precludes the former from entering a body. For the deformable vesicles, one cannot establish an in vivo-in vitro correlation, owing to the mutual effects of the vesicles on transepidermal pathways and vice versa and on the intercellular fluid motion, none of which can be simulated in vitro.

Epicutaneously applied deformable vesicles can ensure a sustained local drug release, in which either the carriers or the involved skin surface acts as a drug reservoir. In turn, even deformable carriers cannot provide a rapidly elevated and high drug concentration in vivo, unless the skin on which the vesicles had been applied is the actual treatment target.

Deformable vesicles and their drug payload applied on skin in a small amount are ‘consumed’ relatively rapidly. They then also deposit drug molecules nearly exclusively near skin surface and in the skin proper. A two to three orders of magnitude higher carrier amount applied on the skin surface tends to favour the systemic drug delivery. Intermediate doses have an intermediate effect. No other system reported in the literature to date offers a similar versatility and controllability of a non-invasive local drug delivery.

3.8 Future Directions and Conclusions

Drug delivery systems cannot be perfected in one step. Further evolution of deformable vesicle (Transfersome®) technology is therefore desirable and likely. This relates to the potential use of self-regulating deformable carriers in medical devices or in gadgets, such as patches, electrically controlled epicutaneous reservoirs, etc. In principle, it should also be possible to equip deformable vesicles with some special feature (a tag, recognition molecule, etc.) to target certain cellular subsets, for example. One future goal is propagation of the positive experiences gained with NSAID targeting into peripheral joints and muscles to other drugs for similar or other peripheral tissues treatment. Another such goal is bringing drug-free therapeutic deformable vesicles into clinical practice. Development of completely non-invasive immunisation involving deformable vesicles as antigen carriers is a further attractive option.

Last but not least, the invention of 3rd-generation deformable vesicles widened the range of deliverable agents. One is now free to use non-phospholipid amphipats, with suitable area per chain, for more versatile deformable vesicle preparation. One has the option to use surfactant-free formulations and to prepare relatively acidic or alkaline dispersions (in which phospholipids would be hydrolysed rapidly). All this opens a number of new perspectives to the deformable vesicle technology. Some will hopefully be pursued actively and lead to the introduction of the corresponding products to peoples' benefit in the not too distant future.

The success of any carrier-mediated transdermal drug delivery relies crucially on good carrier loading on or in the skin, preservation of carrier stability on and in the skin, and carrier adaptability that permits the drug-loaded carriers to enter and move along the narrow conduits in the stratum corneum. Further desiderata are: adequate, and well controlled, drug deposition or release characteristics, once the carrier has crossed the skin barrier through a hydrophilic conduit; existence of drug concentration-independent trans-barrier force (arising e.g. from transepidermal hydration or an electrostatic potential difference), pushing the carrier into skin with sufficient vigour to open trans-barrier conduits and to ensure carriers' adaptation to the resulting pathway dimensions.

The deformable vesicles fulfilling most, if not all, abovementioned requirements are valuable for controlled intra- and transcutaneous drug delivery, as they allow application of otherwise non-deliverable drugs, improve certain drugs performance in vivo, or overcome drug solubility and stability problems.

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Ethosomes: Enhanced Delivery of Drugs to and Across the Skin

4

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Contents

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4.1 Introduction

In spite of the tremendous research carried out in the field of dermal and transdermal drug delivery, efficient administration of drugs by topical application to the skin is still challenging.

In response to the need for efficient delivery of drugs into/across the skin, Touitou has designed a phospholipid vesicular carrier named ethosome (Touitou 1998; Touitou et al. 2000a). This vesicular carrier is distinguished from other lipid nanocarriers by a number of important characteristics, such as the vesicle's bilayer fluidity, its mechanism of permeation enhancement (attributed to the entire system), simple mode of preparation, and lack of side effects. These special features of the ethosomal systems will be further discussed in this chapter.

Since their introduction ethosomes were thoroughly investigated for dermal and transdermal delivery of a variety of actives. Following Touitou and colleagues, other groups have carried out research on the delivery enhancement properties of the ethosomal systems and their safety.

It is noteworthy that in a number of publications describing work with ethosomes, some misleading nomenclature is used, such as elastic liposomes and ethanolic liposomes.

This chapter presents the properties of ethosomes emphasizing some unique features and reviews the work carried out with this vesicular carrier from the beginning till now.

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4.2 Definition, Structure, and the Main Properties of Ethosomes

Ethosomal systems are specially tailored vesicular carriers for enhanced delivery of active agents into the deep layers of the skin and through the skin (Touitou 1998; Touitou et al. 2000a).

Due to the possible interdigitation effect of ethanol on lipid bilayers, it was commonly believed that vesicles cannot coexist with ethanol (Chin and Goldstein 1997; Harris et al. 1987; Pang et al. 1980). Phosphorus nuclear magnetic resonance (PNMR) spectra of ethosomal systems showed a typical configuration of the phospholipid bilayer generally observed in phosphatidylcholine vesicles in water. Furthermore, the paramagnetic-ion NMR spectra indicated that the phospholipid in ethosomes is in a more fluid state and the membrane is more permeable to cations, in comparison to liposomes (Touitou et al. 2000a).

Further confirmation of the fluid nature of ethosomal bilayers was obtained by differential scanning calorimetry (DSC). A comparison of the lipid transition temperatures in ethosomes and in conventional liposomes revealed lower values for the lipids in ethosomes, suggesting that they possess a higher degree of fluidity (Touitou et al. 2000a; Dayan and Touitou 2000; Godin and Touitou 2005). These differences of up to 30 °C in the transition temperatures of ethosomal versus liposomal lipids confirm the fluidizing effect of ethanol on the lipid bilayers in ethosomal systems.

Negatively stained transmission electron (TE) micrographs showed that the ethosomes could vary from unilamellar (Godin and Touitou 2005; Shumilov and Touitou 2010; Shumilov et al. 2010) structures to multilamellar (Touitou et al. 2000a; Godin and Touitou 2004; Ainbinder and Touitou 2005) vesicles with bilayers throughout the vesicle (Fig. 4.1).

The size of the vesicles ranges between 30 nm to several microns and can be adjusted by changes in the system's composition. A systematic investigation of the effect of system composition on vesicular size showed that increase in the percent of lipid results in larger vesicles, while increase in the percent of ethanol at the same lipid concentration decreases the size of ethosomes (Touitou et al. 2000a). This correlation implies that

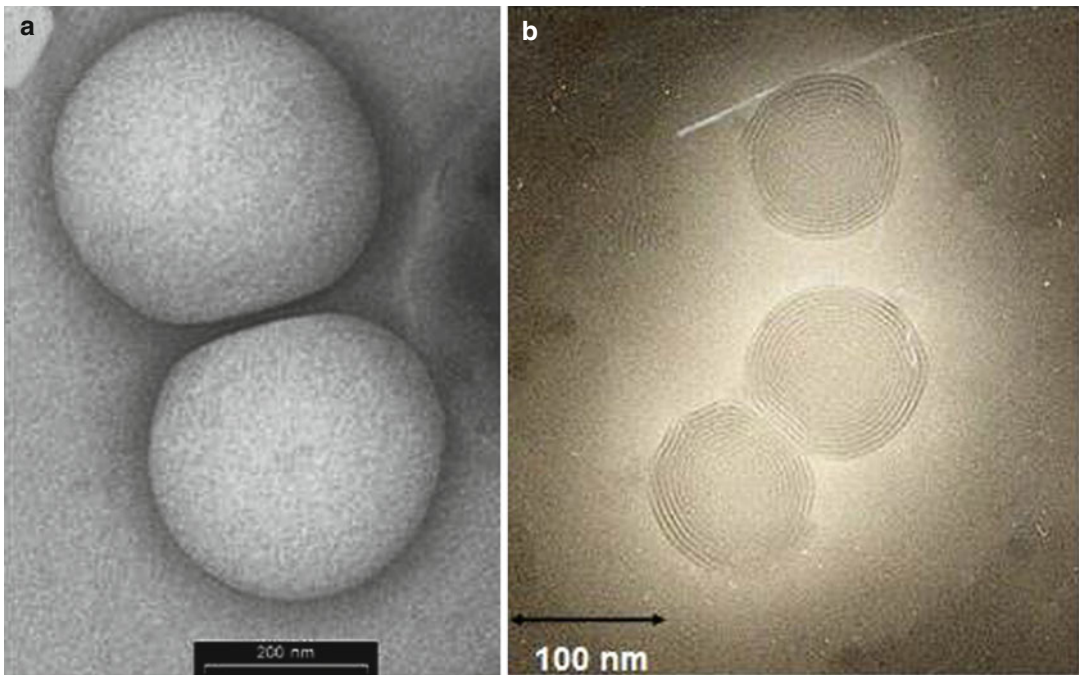


Fig. 4.1 Visualization of uni and miltilamellar ethosomes by TEM

alterations in the concentration of one of the system's components may result in overall modification of the system's characteristics. Therefore, unlike with any other lipid vesicle, in the characterization of ethosomal systems, it is important to use methods adequate for work with soft vesicles. Additionally, special attention should be paid to the dilution of the tested system.

Furthermore, the size of the vesicles in the system is also affected by the vehicle and drug physicochemical characteristics and the concentration of the drug. For example, increase in the concentration of trihexyphenidyl hydrochloride (THP) in the ethosomal system resulted in a decrease in the vesicular size (Dayan and Touitou 2000). Similar effect was observed for bacitracin ethosomal system (Godin and Touitou 2004).

Since the characteristics of ethosomal systems depend on the system composition and ratio between the system components, in their work with ethosomes, researchers should be aware in preserving system's properties. For example, in a number of studies, carried out by researchers without experience in work with ethosomes, the physical characterization protocols were wrong resulting in artifacts and misleading results and conclusions.

An important property of this carrier is its ability to entrap compounds of various hydrophilicities. Visualization of the intra-vesicular distribution of lipophilic (fluorescently labeled phospholipid) and hydrophilic (fluorescein isothiocyanate (FITC)-bacitracin) fluorescent probes by means of confocal laser scanning microscopy (CLSM) showed that both molecules filled the entire volume of ethosomes. These findings were in contrast to those for liposomes, where the lipophilic probe was localized only in the vesicle membrane and the water-soluble FITC-bacitracin filled the core (Godin and Touitou 2004). These results could be explained by the liposomes structure, in which a small number of bilayers surround an aqueous core. The unique structure of ethosomes, due to the presence of ethanol together with vesicle lamellarity, resembles a "fingerprint" in the case of multilamellar vesicles allowing for efficient entrapment of lipophilic and amphiphilic molecules (Touitou and Godin 2005).

Stability of vesicular systems could be evaluated by measuring changes in vesicle size distribution and visualization of the vesicles. The stability of ethosomal systems incorporating various drugs was assessed in a number of studies by comparing the average diameter and the structure of the vesicles during the 2-year period at room temperature (Touitou et al. 2000a; Dayan and Touitou 2000). No changes in the mean size of empty and cationic THP loaded vesicles were observed during the storage interval. Moreover, visualization by negative-stain TEM confirmed that the vesicular structure of the ethosomes persisted after 2 years and no significant structural changes over that time occurred in the systems. In another study with ethosomes (comprising 30 % ethanol, 2 % Phospholipon 90) and the lipophilic antibiotic erythromycin, no significant variations in the vesicle were observed throughout 1-year storage at room temperature (123 ± 15 nm vs. 117 ± 18 nm). TE micrographs confirmed that erythromycin unilamellar ethosomes kept their configuration during the stability evaluation experiment (Godin and Touitou 2005).

Evaluation of the stability of linoleic acid ethosomes by using a Turbiscan optical analyzer showed no modification of the backscattering profiles of colloidal suspensions and no coalescence, sedimentation, flocculation, or clarification (Celia et al. 2009).

Data on SupraVir® cream (Trima, Israel), a marketed ethosomal formulation of acyclovir, indicate that the formulation and the drug have long shelf lives with no stability problems. In this formulation, the drug acyclovir was stable for at least 3 years at 25 °C. Furthermore, skin permeation experiments showed that the initial penetration enhancing capacity of the carrier was retained for at least 3 years (Trima Israel).

4.3 Mechanism of Skin Permeation Enhancement by Means of Ethosomes

The proposed mechanism of permeation enhancement by ethosomes is based on the dual effect of ethanol, on the lipid bilayers in the stratum

corneum (SC) and in the vesicles (Touitou et al. 2000a, b; Touitou and Godin 2005; Godin and Touitou 2003; Ainbinder et al. 2010). The presence of ethanol in ethosome composition enables fluidization of the lipid bilayers in the vesicle together with changes in the arrangement of the lipids in the SC. The ethosome penetrates the altered SC barrier releasing the active agent in the deeper skin layers.

A scheme of the proposed model of penetration enhancement by the ethosomal system through the SC lipids is given in Fig. 4.2. This synergistic effect of the ethanol on the SC lipids and vesicular lipid bilayers was suggested based on the results obtained in fluorescent anisotropy and DSC experiments as well as in skin permeation studies.

In order to gain insights into the characteristics of ethosomes that might allow them to efficiently enhance drug delivery into the skin, free energy measurements of the vesicle bilayers were assessed by DSC and fluorescence anisotropy studies (Touitou et al. 2000a). The transition temperature value of phospholipids in ethosomes was 20–35 °C lower than in liposomes. This can be explained by the fluidity of the phospholipid bilayers in ethosomes (Touitou et al. 2000a; Godin and Touitou 2004). Further confirmation to this fluidity of lipids in ethosomal bilayers was found in fluorescent anisotropy measurements of 9-Antrivinyl labeled analog of phosphatidylcholine (AVPC), where a 20 % lower value was measured in comparison to liposomes (Touitou et al. 2000a).

Skin permeation studies measuring the vesicle components' transport through the skin shed further light on understanding the mechanism of permeation enhancement by ethosomes (Touitou et al. 2000a, b). It was reported that a significant amount (10.5 % of initial) of phosphatidylcholine permeated the skin during a 24-h experiment from an ethosomal system (Touitou et al. 2000a). These results suggest that the vesicles may have entered the skin strata.

Furthermore, skin permeation studies with ethosomes containing a phospholipid fluorescent probe rhodamine red dihexadecanoyl glycerophosphoethanolamine (RR) showed high fluorescence intensity up to a depth of 150 μm (Touitou et al. 2000a). Since this lipophilic probe is used as an indicator of lipid fusion and does not usually cross lipid bilayers, these findings suggest penetration of ethosomes into the deep skin layers.

Experiments with the fluorescently labeled polypeptide, bacitracin (FITC-Bac), showed penetration of the probe through the inter-corneocyte pathways following in vivo application on rat abdomen from ethosomes (Godin and Touitou 2004). The high fluorescence intensity in the intercellular space due to the penetration of FITC-Bac allowed for visualization of clear shadows of hexagonal and pentagonal corneocytes. Examination

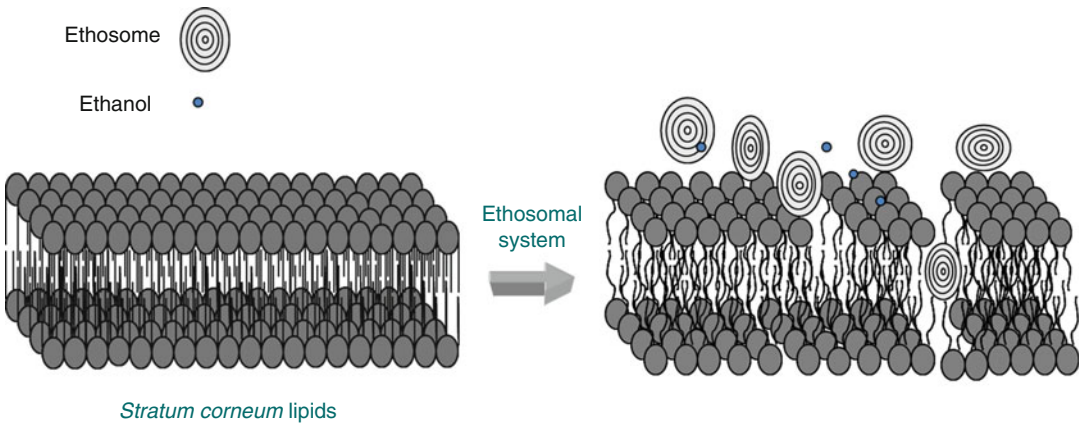


Fig. 4.2 Scheme of the proposed mechanism of enhanced permeation of molecules from ethosomal system across the lipid domain of SC

of the permeation profiles of FITC-Bac from ethosomes through human cadaver skin revealed that occlusion has almost no effect (0.34 ± 0.03 and 0.29 ± 0.02 mg/cm^{2}h for occlusive and nonocclusive conditions, respectively) on skin permeation (Godin and Touitou 2004).

4.4 Ethosomal Systems as Efficient Carriers for Enhanced Drug Delivery into and Across the Skin

4.4.1 Skin Penetration Performance by Ethosomal Carrier: In Vitro Studies

Touitou and her group reported that ethosomes are capable to enhance skin delivery of compounds with a wide spectrum of physicochemical properties as compared to many control systems (Touitou et al. 2000a; Dayan and Touitou 2000; Shumilov and Touitou 2010; Godin and Touitou 2004; Lodzki et al. 2003). Other research groups have further reported the superior skin permeation enhancement ability of ethosomes as compared to various controls, including liposomes and hydroethanolic solutions (Elsayed et al. 2006; Rao et al. 2008; Dubey et al. 2007a, b, 2010; Jain et al. 2007).

The ability of ethosomes (containing 30–45 % of ethanol) to enhance the penetration deep into the skin of lipophilic molecules was first evaluated by using the phospholipid fluorescent probe RR (Touitou et al. 2000a). CLS micrographs of nude mice skin after 8-h application of RR from ethosomes, hydroethanolic solution, and liposomes showed the highest intensity of fluorescence up to a depth of 150 μm with ethosomal systems. No deep penetration of RR from liposomal dispersion was visualized. The application of the hydroethanolic solution containing the same concentration of ethanol resulted in very low fluorescence intensity at the same skin depth as from ethosomes. The results obtained in these experiments, showing delivery of RR to deep skin layers with ethosomes, suggest that ethosomes entered the skin strata to a high depth, in contrast to liposomes, which remained on the skin surface.

One of the first drugs investigated for skin permeation enhancement by ethosomes was THP, a cationic molecule with antimuscarinic activity used for the treatment of Parkinson and dystonia (Dayan and Touitou 2000). Due to the strong evidence that SC is much more permeable to neutral molecules than the salts of weak acids or bases (Swarbrick et al. 1984; Green et al. 1989), delivery into and through the skin of charged molecules is challenging. The results of skin permeation studies carried out in side-by-side diffusion cells showed that THP flux from ethosomes was 87, 51, and 4.5 times higher than from liposomes, phosphate buffer, and hydroethanolic solution, respectively ($p < 0.01$). The quantity of THP in the skin was significantly higher (586 ± 77μg/cm^2, $p < 0.01$) for delivery from ethosomes, while lower amounts were obtained for liposomes (416 ± 27μg/cm^2), hydroethanolic solution (415 ± 21μg/cm^2), and phosphate buffer (127 ± 15μg/cm^2).

Another cationic molecule tested for skin permeation enhancement by ethosomes was buspirone hydrochloride (BH) (Shumilov and Touitou 2010). Delivery of BH from ethosomes (containing 38 % ethanol and 2.5 % phospholipid) across full-thickness porcine ear skin resulted in significantly higher drug amounts in the receiver, relative to drug aqueous solution. The calculated fluxes were 18.77 ± 4.4 and 4.74 ± 0.60 μg/cm^{2}h for ethosomes and aqueous solution, respectively. In order to examine the efficiency of ethosomes in delivery of BH into the skin, a cationic hydrophilic fluorescent probe with a similar molecular weight, rhodamine 6G (R6G), was used as a model. R6G skin penetration versus skin depth profiles showed a significantly higher average fluorescence intensity at various skin depths following application from the ethosomal system ($p < 0.01$). Moreover, increased penetration depth was observed (120 μm vs. 80 μm).

Due to the lipophilic nature of the stratum corneum (SC), highly lipophilic molecules (log $P > 5$) generally show low transdermal absorption. These compounds accumulate within this layer and might encounter problems at the SC–viable

epidermis interface where they must partition into a predominantly aqueous environment. The delivery of a lipophilic drug, minoxidil, into and across the skin was measured after 24-h application of four different compositions all containing 0.5 % w/w of the drug: ethosomal system, phosphatidylcholine ethanolic solution, hydroethanolic solution, and absolute ethanolic solution (Touitou et al. 2000a, b; Godin and Touitou 2003). Delivery of the drug from the ethosomal system resulted in 10, 45, and 35 times higher drug quantity that permeated the skin and 2, 7, and 5 times higher drug quantity accumulated in the skin, compared to phosphatidylcholine ethanolic solution, hydroethanolic solution, or absolute ethanolic solution of the drug, respectively.

Lodzki et al examined the skin permeation of cannabidiol (CBD), a molecule with logP ~8, mediated by an ethosomal carrier (containing 40 % ethanol) (Lodzki et al. 2003). The data from in vitro permeation experiments through nude mice skin indicated that following 24-h application of 100 mg ethosomal composition containing 3 % CBD, a significant amount of the drug permeated the skin (559 μg/cm^2) and CBD skin reservoir (845 μg/cm^2) was generated. This study demonstrated that ethosomes possess the ability not only to enhance the partitioning into the lipophilic layers of the skin but also to enhance the clearance of the drug into the hydrophilic environment leading to transdermal delivery.

Rao and colleagues investigated the percutaneous permeation and skin accumulation of the lipophilic 5α-reductase inhibitor, finasteride, from ethosomal system (Rao et al. 2008). The amount of finasteride permeated through cadaver human skin from ethosomal system was significantly higher ($p<0.01$), as compared to liposomes, aqueous solution, and hydroethanolic solution. Drug transdermal flux from the ethosomal carrier was 7.4, 3.2, and 2.6 times higher than in other formulations. Moreover, higher drug skin accumulation was measured both in the whole skin and in the dermis.

It is well documented in the literature that polypeptides, due to their size and hydrophilicity, generally do not permeate through the intact skin. A work by Godin and Touitou described the ethosomal delivery of FITC-Bac, a polypeptide antibiotic (MW ~ 1.4 kDa), through human cadaver skin. Application of FITC-Bac ethosomes resulted in efficient delivery of the active through the skin with a flux value of around 300 μg/cm^{2}h (Godin and Touitou 2004).

Jain and colleagues have investigated ethosomes for dermal and transdermal delivery of methotrexate (MTX), as an antipsoriatic agent (Dubey et al. 2007a). The steady-state transdermal flux of MTX across human cadaver skin was 57.2 ± 4.3 μg/cm^{2}h, as compared to the significantly lower flux of 14.6 ± 1.6, 22.4 ± 0.2, and 2.2 ± 0.7 μg/cm^{2}h for liposomes, hydroethanolic solution, and drug solution, respectively. Moreover, the lag time following application of MTX ethosomal formulation was the shortest (0.9h), and high skin deposition of the drug was obtained (31 % vs. 8, 6, and 2 in other formulations, respectively).

Similar superior skin permeation enhancement and deposition of ethosomes was observed with melatonin (Dubey et al. 2007b). The transdermal flux through human cadaver skin for melatonin-loaded ethosomes was found to be 59.2 ± 1.2 μg/cm^{2}h versus 22.4 ± 0.2 μg/cm^{2}h for hydroethanolic solution and 10.9 ± 1.6 μg/cm^{2}h for liposomes.

Enhanced transdermal delivery of lamivudine from ethosomes was reported by Jain and colleagues (Jain et al. 2007). In their study, 5-, 8-, 12-, 15-, and 25-fold higher drug skin flux from ethosomes was obtained as compared to liposomal formulation, phospholipid solution in ethanol, hydroalcoholic solution, and PBS solution.

In a recent study, the evaluation of the effect of ethosomes on transdermal permeation of the anti-HIV agent, indinavir, carried out by Jain and his group demonstrated again the superior effect of this carrier (Dubey et al. 2010). At least two times higher transdermal flux of the drug was obtained from ethosomal system as compared to drug hydroethanolic solution, liposomes, and aqueous solution. Drug-loaded ethosomes facilitated better skin drug deposition (28.3 %) as compared with ethanolic solution (16.1 %), liposomes (11.2 %), and drug solution (4.6 %).

The results summarized above show superior skin permeation enhancement by the ethosomal system as compared to ethanol, hydroalcoholic solutions with same ethanol concentration as in ethosomes, ethanolic phospholipid solutions, or classic liposomes. The enhanced skin permeation effect was shown for molecules with various properties, including lipophilic, charged, and large molecular weight (MW) actives.

4.4.2 Proof of Concept In Vivo and in Clinical Trials

Since their invention, many pharmacokinetic and pharmacodynamic studies on animals and in humans were carried out with ethosomal systems. Some are further overviewed categorized by their therapeutic use (Touitou et al. 2000a, 2007; Godin and Touitou 2005; Shumilov and Touitou 2010; Shumilov et al. 2010; Ainbinder and Touitou 2005; Lodzki et al. 2003; Godin et al. 1999, 2005; Horwitz et al. 1999; Chertin et al. 2006; Paolino et al. 2005; Dkeidek and Touitou 1999; Meidan and Touitou 2001).

4.4.2.1 Skin Infections Treatment with Ethosomal Systems

Erythromycin ethosomal systems (containing 30 % ethanol and 2 % Phospholipon 90) were investigated in the treatment of various skin infections (Godin and Touitou 2005; Godin et al. 2005). Measurement of the pharmacodynamic effect in immunocompetent Hsd:ICR (CD-1®) male mice (Harlan, Israel) injected intradermally with *Staphylococcus aureus*, by isolation of *Staphylococcus aureus* colonies from the skin wounds, showed that no bacteria were found in the inoculation sites in mice treated with ethosomal erythromycin system compared to 0.90×10^7 and 0.57×10^7 cfu/g tissue on days 7 and 10 after the beginning of the experiment in untreated mice. Histological examination of the wounded skin tissue revealed no dermatonecrosis and preservation of normal skin structures. Wounded areas from untreated mice and mice treated with erythromycin hydroethanolic solution showed progression of the infection, resulting in significant necrosis of the skin and adjacent tissues and initial crust formation over the necrotic area (Fig. 4.3). These findings demonstrate the ability of erythromycin ethosomal

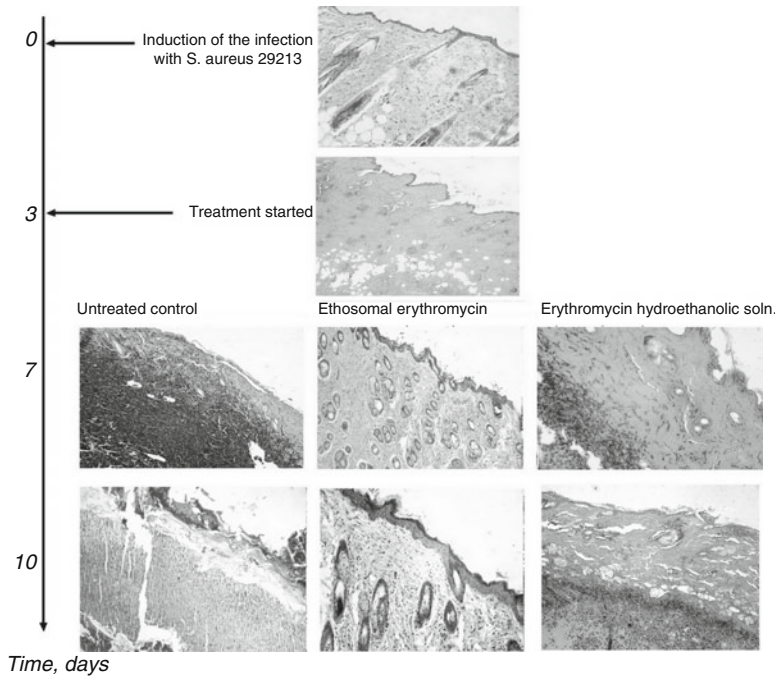


Fig. 4.3 Histological images taken from skin of mice intradermally inoculated with 0.1 mLx10^8 cfu/mL (10^7 cfu/mouse) *S. aureus* ATCC 29213 on days 0, 3, 7 and 10 after challenge. Mice groups: untreated control (*left panels*), ethosomal erythromycin applied on the infected skin (*middle panels*) and hydroethanolic erythromycin solution applied on the infected skin (*right panels*). (From Godin et al. 2005, with permission from Oxford Journals.)

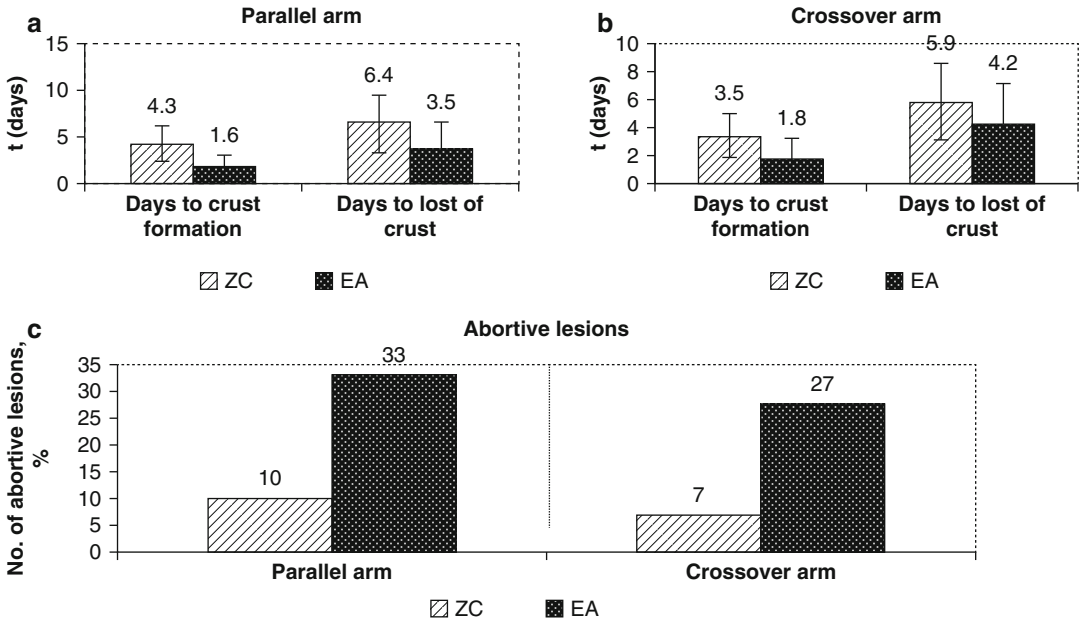


Fig. 4.4 Parameters assessed in a two-armed, randomized, double-blind clinical study in recurrent herpes labialis patients with two formulations containing 5 % acyclovir: ethosomal acyclovir (EA) versus Zovirax cream (ZC) (From Horwitz et al. 1999, with permission from Elsevier)

system to eradicate the bacteria at the site of inoculation in the deep skin strata, thus suggesting this system as a good alternative to systemic administration of the drug by injection in the treatment of deep skin infections.

A pilot clinical study was carried out to test the efficacy of a new clindamycin phosphate and salicylic acid (CLSA) ethosomal gel for the treatment of mild to moderate acne vulgaris (Touitou et al. 2007). In this study, carried out on 40 patients, the efficacy of CLSA ethosomal gel in the treatment of mild to moderate acne vulgaris was investigated. A considerable improvement of the acneic condition, significantly decreasing the number of comedones, pustules, and total number of lesions, was found following twice-a-day treatment with CLSA ethosomal gel during 8 weeks. Seventy-one percent of the participants indicated improvement of their condition, with no reports on worsening. Furthermore, 14 of the 17 participants with a history of previous topical treatment preferred the CLSA ethosomal gel over prior commercial topical medications, based on improved tolerability and fewer side effects.

An ethosomal system containing the synthetic acyclic nucleoside analog, acyclovir (ACV), was designed and tested for the treatment of herpes labialis (Horwitz et al. 1999). In a randomized double-blind clinical study, the efficiency of ACV ethosomal formulation was compared to a commercial acyclovir cream (Zovirax®, GlaxoSmithKline) and a solution of the free drug (Fig. 4.4). The parallel arm consisted of 31 participants of whom 12 received ethosomal acyclovir (EA), 10 Zovirax® cream (ZC), and 9 vehicle (V). In the crossover arm, 8 participants were treated with EA followed by ZC and 7 participants were treated with ZC followed by EA. Application of EA system resulted in a significant improvement of time (days) to crust formation, time (days) to loss of crust, the proportion of abortive lesions of all assessable lesions, time (days) to first reduction of reported pain intensity, time (days) to absolute resolution of pain, and the proportion of lesions in which reported pain intensity was reduced from day 1 to 2 and from day 1 to 3. Comparison with ZC showed that in the parallel arm on the third day from the begin-

ning of herpetic episode, 80 % of lesions crusted after treatment with EA versus only 10 % in the ZC group. The time to crust formation was 1.6 days in the EA group versus 4.3 and 4.8 in the ZC and V groups, respectively. Moreover, 33 % of the lesions in the EA group were abortive compared to only 10 % in the ZC group. In the crossover arm, the number of days to crust loss was significantly reduced in the EA group from 4.2 to 5.9 in ZC group on day 2. Sixty percent of the lesions in the EA group crusted versus only 15 % of lesions treated with ZC. The findings of this clinical study showed improved clinical efficacy of ethosomal acyclovir compared to ZC and lead to the release of a new topical acyclovir cream based on the ethosome technology, the SupraVir (Trima, Israel)

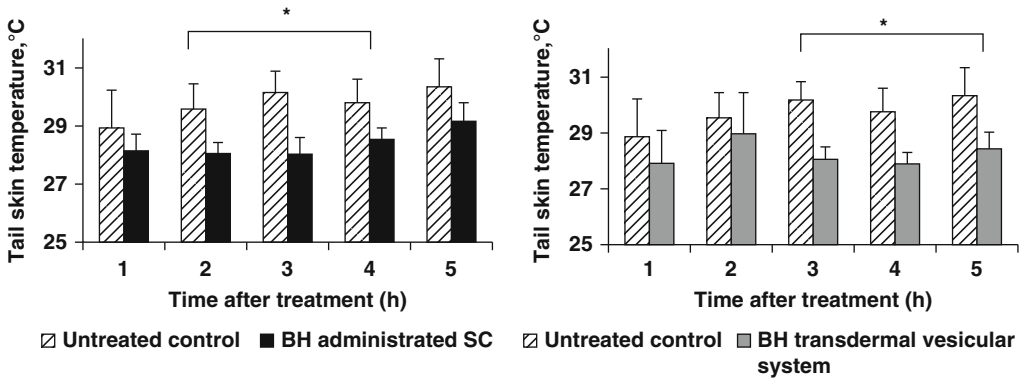
4.4.2.2 Hypogonadal and Menopausal Syndromes and Erectile Dysfunction Transdermal Treatments with Ethosomal Systems

Transdermal delivery of testosterone from an ethosomal patch named Testosome was compared to Testoderm® patch (Alza, Palo Alto, CA, USA), in an in vivo study on rabbits following daily applications for five consecutive days (Touitou et al. 2000a, b). At least twice higher area under the concentration-time curve (AUC) and the highest concentration measured at any time (Cmax) values were calculated for the ethosomal system following the collection of blood samples and their analysis by radioimmunoassay at the end of the experiment.

In a later study in rats, plasma testosterone levels following topical application from ethosomal formulation were measured (Ainbinder and Touitou 2005). Significantly higher ($p < 0.05$) Cmax and AUC values for ethosomal formulation were obtained as compared to a commercially available preparation, AndroGel® (Unimed, USA). Skin permeation studies through human cadaver skin enabled to calculate the flux of the drug through the skin ($1.24 \times 10^{-2} mgh^{-1}/cm^{-2}$). Theoretical calculation of testosterone plasma concentrations expected in humans showed that application area of 40 cm^2 would be sufficient to achieve plasma values within the normal hormone range in men. These results show that ethosomes allow for enhanced in vivo transdermal absorption of the steroid hormone testosterone enabling to achieve therapeutically efficient plasma levels.

Another investigation was carried out for BH ethosomal system in the treatment of hot flashes, the most common menopausal syndrome in women (Shumilov and Touitou 2010). The authors studied the pharmacodynamic effect in both hot flashes and anxiety animal models. For hot flashes, a decrease in the elevated tail skin temperature (TST) was measured in the estrogen deficiency-associated thermoregulatory dysfunction rat model, produced by bilateral ovariectomization (OVX rats) (Berendsen et al. 2001). Application of BH ethosomal system on the skin of OVX rats caused a decrease in the elevated tail temperature 3 h after administration, which continued for a total period of 6 h, until the end of the experiment (Fig. 4.5). A faster (2 h) but shorter (3 h) decrease in TST was obtained following subcutaneous injection of the drug. Pharmacokinetic studies have shown a prolonged presence of the drug in the rat plasma following application of the transdermal BH ethosomes as compared to oral administration. These findings suggest that transdermal delivery of BH from the ethosomal system may result in a continuous delivery of BH into the bloodstream, possibly offering sustained efficacy with reduced side effects.

The effect of prostaglandin E1 (PGE1) ethosomal systems on erectile dysfunction was tested in an "in-office" pilot clinical study, carried out on 16 men with 17 episodes of erectile dysfunction (Chertin et al. 2006). Patients, treated with ethosomal systems topically applied on the glans penis, were asked to evaluate their ability to have sexual intercourse by scoring the erectile response, in addition to erection assessment by a physician. Furthermore, duplex examination of the cavernous arteries 15 min following the application, in order to assess peak systolic velocity (PSV) and pulsative index (PI) of both left and right cavernous arteries, was measured. The results show that following a single topical application of PGE1 ethosomal system, enhanced penile rigidity and improved peak systolic



	Dose	Onset	Duration	Mean ΔTST	Max ΔTST
Buspirone administrated SC	5	2	3	1.47 ± 0.5	2.1
Buspirone transdermal ethosomal system	15	3	>3	1.6 ± 0.7	2.1

Fig. 4.5 Effect of buspirone (BH) following: (A) subcutaneous (n=4) and (B) transdermal administration (n=5) on elevated TST in OVX rats. TST are measured in the active phase 1, 2, 3, 4, and 5 h after treatment (*TST* tail skin temperature; *OVX* ovariectomized). Mean ± S.D.; **p<0.05* compared to untreated OVX group (From Shumilov and Touitou 2010, with permission from Elsevier)

velocity were observed in 12 out of 15 patients. The duration of erection varied between 10 and 60 min. Empty ethosomal system had no effect on the erectile response or the penile blood flow. No penile erythema or other adverse events have been reported by any of the participants in any of the study groups. The results of this pilot study show that topical application of PGE1 ethosomal systems could be a promising approach for the local treatment of erectile dysfunction.

4.4.2.3 Anti-inflammatory, Antipyretic, and Analgesic Treatments with Ethosomal Systems

Cannabidiol (CBD) is a potent anti-inflammatory agent in the treatment of rheumatoid arthritis (Malfait et al. 2000). Lodzki and colleagues have studied the anti-inflammatory potential of transdermal ethosomal CBD system (Lodzki et al. 2003). A significant accumulation of the drug in the skin and in the underlying muscle was measured following a 24-h application of CBD ethosomes to the abdominal skin of nude mice. CBD was detected in the hip skin (37.43 ± 13.58 μg/cm^2), abdominal skin (110.07 ± 24.15 μg/cm^2), and abdominal muscle (11.54 μg CBD/g muscle), as well as in the hip muscle, liver, and pancreas. Drug plasma concentrations measured during 72-h system application in ICR mice indicated that steady-state levels of the drug (0.67 μg/ml) were achieved after 24 h and lasted until the end of the experiment (72 h). The anti-inflammatory effect of ethosomal CBD system was evaluated using carrageenan-induced aseptic paw inflammation in ICR mice (Sammons et al. 1997). In these experiments, the effect was measured as delta calculated by deduction of the thickness of saline-injected paw from that of the carrageenan-injected inflamed paw in each mouse. Hourly measurements of mice paw thickness for up to 4 h demonstrated a significant difference in the pharmacodynamic profiles of CBD treated and untreated animals, indicating that the inflammation was prevented by transdermal delivery of ethosomal CBD (Fig. 4.6). These results show that efficient delivery of CBD can be achieved by transdermal administration from ethosomal system, enabling to overcome the drawbacks

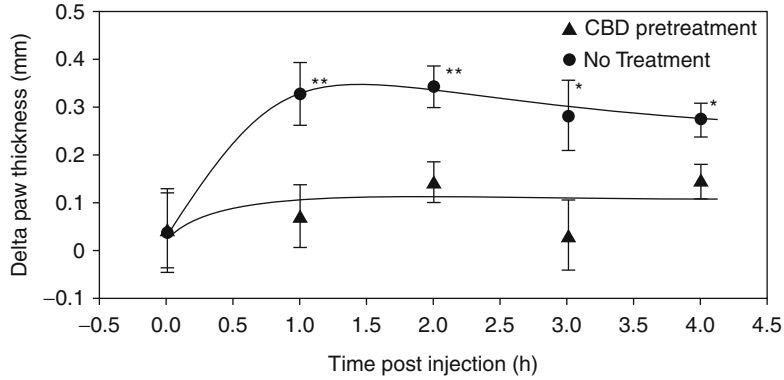


Fig. 4.6 Anti-inflammatory effect of CBD transdermal ethosomal patch, applied 19 h prior to the injection, is compared to no pretreatment: **Δ** (mean±S.E.M.) between the thickness of carrageenan injected and saline injected paws of the same mouse at different time points post injection. $^{**}p<0.01$; $^{*}p<0.05$ (From Lodzki et al. 2003, with permission from Elsevier)

associated with oral drug delivery, including low oral bioavailability, extensive first-pass hepatic metabolism, instability in the acidic gastric pH, and/or low water solubility.

Paolino and colleagues have evaluated the anti-erythemal efficacy of a natural anti-inflammatory agent, ammonium glycyrrhizinate (AG), delivered from ethosomes (containing 1–3 % Phospholipon 90 and 30–45 % ethanol) versus same concentration ethanolic solution and aqueous solution, in humans (Paolino et al. 2005). The research was carried out on 12 healthy volunteers pretreated with AG systems 1, 3, and 5 h prior to application of methyl nicotinate (MN), a vasodilator agent, and tested the induced erythema at six sites on the ventral surface of each forearm. A significant reduction in the intensity and duration of erythema, monitored for 8 h by using a reflectance visible spectrophotometer, was induced by ethosomal AG. The maximal erythema index (ΔE.I.) following 5-h pretreatment with AG ethosomal system was 29.6 % versus 62.7 and 60.7 %, for ethanolic and aqueous controls, respectively. No erythema was observed in sites treated with AG ethosomes 3 h after topical application, while sites treated with aqueous or hydroethanolic solutions of the drug showed presence of chemically induced erythema. It is noteworthy that an examination of the possible effect of empty ethosomes showed no anti-inflammatory activity. These results show that pretreatment of the skin with ethosomal AG system for 1, 3, and 5 h was able to antagonize the appearance of the erythema.

A recent study by Touitou and her group investigated the analgesic and antipyretic therapeutic effects of transdermal ethosomal ibuprofen system in two animal models, the Brewer's yeast-induced fever rat and tail flick nociception mice (Shumilov et al. 2010). Application of transdermal ethosomal ibuprofen gel resulted in a gradual decrease in the body temperature of fevered rats, achieving normal values within 3 h (37.0 ± 0.2 °C) and remaining low for at least 12 h. The oral administration resulted in rat's body temperature returning to baseline after 1 h, but remaining low for only 7 h and followed by a rise to 38.0 ± 0.4 °C. A significant ($p < 0.05$) analgesic effect of ethosomal ibuprofen gel 120 and 360 min after administration compared to oral treatment was observed by tail flick test in mice. The data obtained in this work suggest that ethosomal ibuprofen gel might allow for an efficient antipyretic and analgesic treatment, with continuous drug input to the systemic circulation and avoidance of possible gastrointestinal ulceration and bleeding. Moreover, transdermal ibuprofen gel could be beneficial for pediatric patients who often refuse to take the full dose of the medication orally or suffer from vomiting.

4.4.2.4 Insulin Delivery with Ethosomal Systems

Transdermal delivery of proteins from the ethosomal system was tested by measuring the effect of insulin on lowering blood glucose levels (BGL) (Touitou et al. 2000b; Dkeidek and Touitou 1999; Dkeidek 1999). Insulin delivered transdermally from an ethosomal patch caused a significant reduction (up to 60 %) in BGL in both normal and diabetic rats. On the other hand, insulin skin application from a control non-ethosomal formulation was not able to decrease the blood levels of glucose. The results of this study show that the ethosomal system enables an efficient delivery of insulin through the skin resulting in a significant pharmacodynamic response. The prolonged plateau effect, lasting for at least 8 h, demonstrates the advantage of ethosomes for transdermal delivery of insulin.

4.4.2.5 Pilosebaceous and Hair Follicle Treatments with Ethosomal Systems

Efficient delivery of actives to pilosebaceous and hair follicular units could highly improve therapies targeting skin appendage-related disorders such as seborrhea, hair loss, and acne. In order to test the efficiency of ethosomes in delivering drugs to skin appendages, minoxidil, a lipid-soluble drug administered topically on the scalp for alopecia treatment, was incorporated into ethosomes, and the system was evaluated for localization of the drug into the pilosebaceous units (Meidan and Touitou 2001). Localization of H^3-minoxidil within the pilosebaceous units was observed (Godin and Touitou 2003; Touitou et al. 1998, 2000b) and measured by quantitative skin autoradiography (Fabin and Touitou 1991; Godin et al. 1999) following the application of compositions containing 0.5 % minoxidil and 50 μCi tritiated drug to the dorsal region of hairless rats for up to 24 h. Comparison to liposomes demonstrated that the ethosomal system was superior in delivery of minoxidil to the pilosebaceous units (22 vs. 4.5 nmol/g tissue, $p < 0.005$) (Godin and Touitou 2003; Touitou et al. 1998, 2000b). Thus, administration of minoxidil from ethosomal system could result in drug targeting to the hair follicles and improvement of the low efficiency of the current minoxidil therapy for alopecia.

The findings of the studies reported above suggest that ethosomes are able to efficiently enhance skin permeation of various molecules leading to improved dermal and transdermal treatments of deep skin and systemic ailments.

4.5 Safety Evaluation In Vitro, in Animals, and in Human Studies

Many penetration enhancers besides interfering with the barrier function of the skin cause also damage to the skin cells and induce local irritation. In contrast to these materials, ethosomal carriers composed of inactive ingredients for pharmaceutical use approved by FDA enable efficient skin permeation enhancement on a safe mode.

The safety of ethosomal systems was tested in numerous works, both in vitro and in vivo (Fig. 4.7). Some of them are outlined below.

An in vitro live/dead viability/cytotoxicity viability test carried out in cultured fibroblasts for various vesicular systems and controls indicated that the ethosomal carrier (containing 2 % Phospholipon 90 and 30 % ethanol) is not toxic to 3T3 fibroblasts and that cultured cells kept their viability.

Studies in animals have evaluated the safety of ethosomes to the skin following single or chronic application (Shumilov and Touitou 2010; Shumilov et al. 2010; Ainbinder and Touitou 2005; Lodzki et al. 2003; Dayan and Touitou 2000). Histological observations of the skin at the site of treatment showed no changes in the skin structure and in the thickness of the horny layer and no infiltration of inflammatory cells to the skin. Furthermore, biochemical analysis of rat's blood after 5-day treatment with transdermal ethosomal ibuprofen gel revealed no statistically significant differences between the treated and the control groups (Shumilov et al. 2010).

Paolino and Fresta carried out a study testing the skin tolerability of ethosomal systems in

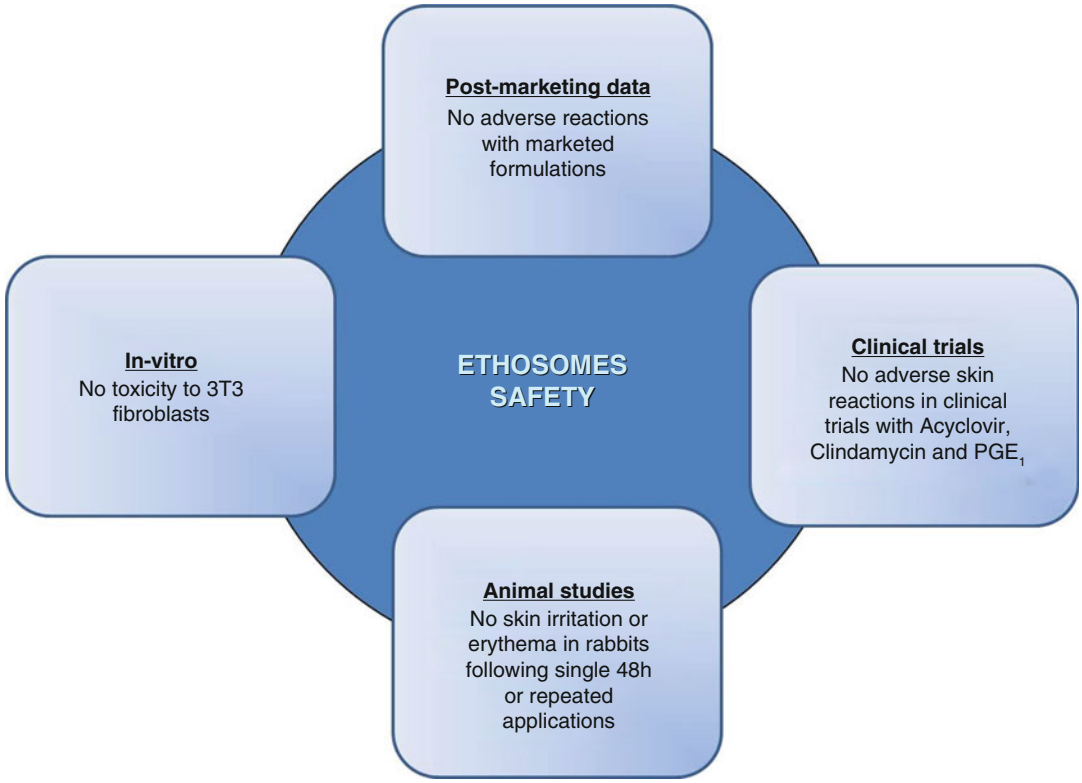


Fig. 4.7 Safety studies with ethosomes

human volunteers utilizing a noninvasive technique of reflectance spectrophotometry (Paolino et al. 2005). No signs of erythema following 12-, 24-, or 48-h application of ethosomal carrier containing 2 % Phospholipid and 45 % ethanol were reported. In contrast to this, application of hydroethanolic solution with an equal water/ethanol ratio to that of ethosomes resulted in significant skin erythema. Moreover, no significant difference in erythema index (ΔEI) was measured between skin areas treated with ethosomes and saline.

In three separate studies in humans, application of ethosomal systems containing clindamycin, acyclovir, or PGE_1 to the skin of human volunteers has shown no adverse skin reactions, and products based on ethosomal carriers have been marketed for a number of years without any reports on skin irritation or safety issues.

4.6 Summary

Ethosomes are phospholipid vesicular systems for dermal and transdermal delivery of actives. The proposed permeation enhancement mechanism suggests that ethanol present in the system has a fluidizing effect both on the phospholipid bilayers of the vesicle and on the SC lipid bilayers of the intercellular pathway in the skin. The vesicle penetrates through the disorganized SC lipids into the deep skin strata where it releases its contents. In vitro, in vivo, and clinical studies summarized in this chapter show that this passive delivery system is able to efficiently enhance skin penetration of molecules with various physicochemical characteristics and structures. In terms of safety, no local irritation was detected following skin application of ethosomes. The enhanced penetration of drugs deep into and across the skin

by means of ethosomal carrier could be valuable in a variety of existing and new emerging therapies.

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Invasomes: Vesicles for Enhanced Skin Delivery of Drugs

5

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Contents

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5.1 Introduction

The major problem in both topical and transdermal drug delivery is the low permeability of the most apical layer of the skin, the stratum corneum (SC). Due to this, topical formulations encounter the problem of inefficient penetration of drugs into the skin which results in a low dose reaching the site of action and therefore limited local activity. Transdermal drug delivery, i.e., drug absorption into systemic circulation, is even more difficult to achieve, and currently, only a limited number of drugs with low molecular weight (MW), such as scopolamine, clonidine, nitroglycerin, fentanyl, nicotine, estradiol (alone or in combination with levonorgestrel or norethisterone), and some others are used in transdermal patches (Benson 2005; Prausnitz and Langer 2008). Therefore, in order to penetrate easily the SC, a molecule should posses certain physicochemcial properties, such as low MW (<600 Da), adequate solubility in oil and water, intermediate partition coefficient (log *K* octanol/water of 1–3), and low melting point (Barry 2001; Williams 2003).

If the drug does not match these ideal characteristics, different penetration enhancement techniques can be used to overcome the barrier properties of the SC. These methods include manipulation of the drug or vehicle to enhance drug diffusion (Barry 2004), use of nanocarriers (Benson 2005), as well as methods which modify

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the SC, such as iontophoresis (Lopez et al. 2003), electroporation (Wang et al. 2009), ultrasound (Cancel et al. 2004), and chemical penetration enhancement using different penetration enhancers (Ahad et al. 2009). The successful use of these methods is well documented in literature (Barry 2001; Benson 2005; Rizwan et al. 2009) and is described in detail in separate chapters of this book.

Among these different methods, nanocarriers have been frequently used, especially liposomes (Cevc et al. 2008a, b; Song and Kim 2006). Liposomes have been intensively studied as drug carrier systems for both the topical (dermal) and transdermal drug delivery (Cevc and Blume 2001, 2004; Cevc 2003; El Maghraby and Williams 2009).

5.2 Liposomes

Liposomes are used as topical drug delivery systems in dermato-pharmacotherapy, as there is a need for a drug delivery system that enhances penetration of the active ingredient into the skin, localizes the drug at the site of action, and reduces percutaneous absorption. Liposomes indeed have the potential to enhance drug penetration into the skin (El Maghraby et al. 2001a; Verma et al. 2003a, b; Betz et al. 2005), improve therapeutic effectiveness (Seth et al. 2004; Mura et al. 2007), decrease side effects (Seth et al. 2004), and act as a local depot for sustained release of dermally active components (Schreier and Bouwstra 1994). For achieving these localizing drug effects in the skin, conventional liposomes typically composed of phospholipids and cholesterol are used. For a review of studies reflecting the localizing effect of liposomes, which is used in local skin treatments with corticosteroids, antimycotics, local anesthetics, and others, the reader should refer to Chap. 2 in this book.

As it has been generally agreed that conventional liposomes are of little or no value as carriers for transdermal drug delivery, because they do not deeply penetrate the skin, but rather remain confined to the upper layer of the SC, there was a need for the development of more potent novel vesicles, which could potentially be used as transdermal drug delivery systems. Thus, these novel vesicles should be used for drug delivery into the deepest skin layers of the SC (dermal effect) or the subcutaneous tissue (regional effect) or the systemic circulation (transdermal effect) dependent on the indication. In order to develop such vesicles, the lipid composition of liposomes was modified. As the key parameter affecting drug permeation across the SC and the interactions with the SC is the thermodynamic state of the liposomes' bilayers which depend on their lipid composition (i.e., the transition temperature (Tm) of their lipids), the latter was modified. Bilayers may be either in a liquid crystalline state, which is characterized by the fluid state of bilayers, or in a gel thermodynamic state, characterized by rigid bilayers (Riaz et al. 1989). Since conventional liquid-state vesicles have proven to be superior to gel-state vesicles (Van Kuijk-Meuwissen et al. 1998a, b; El Maghraby et al. 1999, 2001a), while elastic vesicles have been shown to be superior to conventional gel-state and even liquid-state vesicles in terms of interactions with human skin (van den Bergh et al. 1999) and enhanced drug penetration (El Maghraby et al. 1999), a series of liquid-state vesicles with elastic membranes were developed.

The first elastic or deformable vesicles, termed Transfersomes® (Idea AG, Germany), were introduced in the 1990s by Cevc et al. (1995). These vesicles contained phosphatidylcholine (PC) and edge activators (sodium cholate, polysorbate 80, or polysorbate 20) to impart deformability to the carrier, being responsible for improved dermal/transdermal drug delivery (Cevc et al. 1998; 2002; 2008a, b, c; Cevc and Blume 2001, 2003, 2004). These vesicles provided a tenfold higher diclofenac amount in the subcutaneous tissue compared to a commercial diclofenac gel (Cevc and Blume 2001), as well as a higher ketoprofen accumulation in deep subcutaneous tissues with Diractin® (gel with ketoprofen-loaded Transfersomes®) compared to conventional gels (Gabrilen® gel, Togal® Mobil Gel, Fastum® gel) (Cevc et al. 2008c). Further, *in vivo* studies in mice and humans revealed that Transfersomes® enabled a systemic delivery of insulin and that

the efficiency of the formulation was comparable to that obtained after a subcutaneous (s.c.) injection of the same preparation, but with a longer lag time (Cevc et al. 1995, 1998).

Based on the positive results obtained with Transfersomes®, a series of novel deformable vesicles was developed by modifying the composition of vesicles. These vesicles include ethosomes, vesosomes, penetration enhancer-containing vesicles (PEVs), invasomes, etc. (Touitou et al. 2000; Verma 2002; Mishra et al. 2006; Mura et al. 2009; Mura et al. 2011). The aim of this chapter is to introduce invasomes as novel nanocarriers, and for other vesicles, one should refer to other chapters in Part 2 of this book.

5.3 Invasomes

5.3.1 Development of Invasomes

Invasomes (as termed by the inventors) were introduced by the group of Professor Alfred Fahr (Verma 2002). They were composed of unsaturated soybean lecithin (with high % PC), small amount of ethanol, and small amount of a mixture of terpenes (cineole, citral, and d-limonene). Unsaturated phospholipids were chosen as they, due to their low Tm, lead to the formation of liposomes being in liquid crystalline thermodynamic state. The purpose of using terpenes was to impart deformability to the carrier. It was supposed that terpenes, which are used as penetration enhancers, as they increase the fluidity of SC lipid bilayers (Cornwell et al. 1994), would also increase the fluidity of vesicles' bilayers. Namely, terpenes have been shown to be potent enhancers for a variety of drugs, such as nicardipine (Krishnaiah et al. 2003), lorazepam, clonazepam (Puglia et al. 2001), haloperidol (Vaddi et al. 2002), nicardipine, carbamazepine, tamoxifen (El-Kattan et al. 2001), etc. Investigations employing differential scanning calorimetry (DSC) and x-ray diffraction revealed that terpenes increase drug permeation by disrupting lipid packaging of the SC and/or disturbing the stacking of the bilayers (Cornwell et al. 1994). Moreover, the enhanced skin penetration of various drugs is supposed to be a result of increased drug solubility in the SC treated by terpenes. The lipophilic drugs show increased penetration due to their increased partition coefficient SC/vehicle, and their penetration increases proportionally to their solubility in the enhancer. Regarding hydrophilic drugs, their penetration is assumed to be improved due to their increased diffusion coefficient (Williams and Barry 1991a, b; Cornwell et al. 1996; Moghimi et al. 1997). Ethanol was also added to liposomes, besides the assumption that ethanol is detrimental to liposomes and is therefore removed from ethanolic solutions of phospholipids during the preparation of liposomes. Ethanol was added as it was believed that it would fluidize the vesicles' bilayers in the same manner as it fluidizes the SC lipid bilayers. This assumption was already confirmed by Touitou et al. (2000) who developed ethosomes – vesicles containing besides phospholipids and water also high amounts of ethanol (>30 %) – which have proven to be able to deliver drugs to the deep skin layers and/or the systemic circulation (Godin and Touitou 2003; Ainbinder and Touitou 2005; Dubey et al. 2010). Hence, it has been shown that ethanol is a potent penetration enhancer not only in combination with other chemical or physical penetration enhancers (Srinivasan et al. 1990; Kobayashi et al. 1994; Bhatia and Singh 1999), but also in combination with liposomes due to their synergistic effect (Touitou et al. 2000; Verma and Fahr 2004).

In conclusion, the inventors of invasomes assumed that these potent penetration enhancers, ethanol and terpenes, would act synergistically on the fluidity and deformability of the vesicles' bilayers, as well as on disturbing the SC lipid bilayers. Further, these enhancers could act synergistically with liposomes in enhancing the drug penetration into the skin.

5.3.2 Penetration-Enhancing Ability of Invasomes

Verma (2002) performed the first studies with invasomes using cyclosporine A (CsA, CyA) as a drug. The aim was to enhance the penetration of

CsA into the skin, being a challenge as CsA does not possess favorable physicochemical properties to penetrate the skin. CsA is a lipophilic drug with MW of 1202.61 Da and a partition coefficient of 4000 and, hence, shows a poor penetration into the skin. The topical use of CsA would be advantageous in treating psoriasis and dermatological diseases that are thought to have an inflammatory T-cell-mediated pathogenesis. Further, due to its stimulating effect on hair growth, CsA has been considered for the treatment of alopecia areata, as well as for the treatment of androgenetic alopecia.

Verma prepared CsA-loaded invasomes containing 10 % w/v unsaturated soybean PC; 3.3 % w/v ethanol; 0.5, 1.0, and 1.5 % w/v of a terpene mixture composed of citral, cineole and d-limonene (cineole:citral:d-limonene = 45:45:10 v/v = standard terpene mixture); and phosphate buffer saline (PBS) up to 100 % w/v. *In vitro* studies in human skin revealed (Fig. 5.1) that invasomes provided a significantly higher amount of CsA in the deeper layers of human skin (viable epidermis and dermis) compared to conventional liquid-state liposomes (without ethanol and terpenes) and the aqueous/ethanolic drug solution (Verma 2002). In addition, increasing the amount of terpenes from 0.5 to 1.5 % increased the amount of CsA recovered in the SC and deeper skin layers, indicating a direct correlation between the amount of added terpenes and the amount of drug found in the skin. However, there was no statistically significant difference between invasomes containing 1 or 1.5 % standard terpene mixture. This study proved the penetration-enhancing ability of invasomes and revealed that invasomes present an effective carrier system for delivering the highly lipophilic CsA to the deeper skin layers where it should exert its therapeutic effect.

Invasomes have also been investigated for their influence on the skin delivery of temoporfin (mTHPC), which is an interesting candidate for topical photodynamic therapy (PDT) of cutaneous malignant and nonmalignant diseases. Unfortunately, mTHPC (Dragicevic-Curic et al., 2008a) like CsA possesses unfavorable properties to easily penetrate the skin. It has a MW of 680 Da and is highly hydrophobic (octanol/water partition coefficient of 9.4) (Kelbauskas 2003).

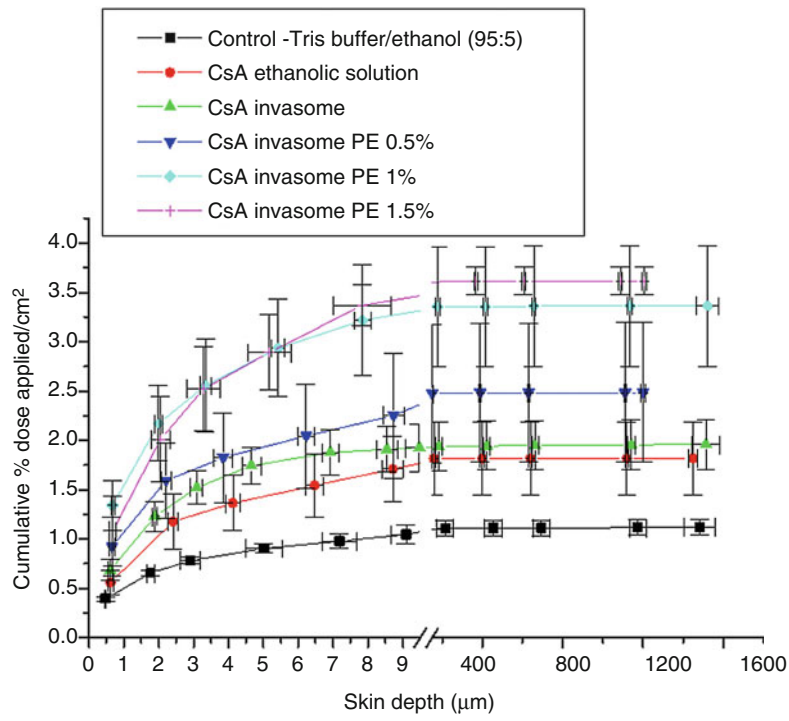

Fig. 5.1 Skin depth profile of CsA after 6 h nonocclusive application of different formulations onto human abdominal skin (expressed as cumulative % of dose applied/cm^2 ± SE, $n = 3–6$). *PE* penetration enhancer, i.e., standard terpene mixture (Verma 2002)

Consequently, mTHPC exhibits low percutaneous absorption and there are no topical formulations with mTHPC at the market. It has been to date applied only intravenously in PDT of skin cancers (Kübler et al. 1999). Hence, it was very important to develop a formulation enabling the skin delivery of mTHPC, i.e., enhancing its penetration into the skin and depositing sufficiently high amounts of mTHPC in the deeper skin layers, which would ensure a positive outcome in PDT of different skin diseases. The topical use of mTHPC would simplify PDT, since the skin is readily accessible for topical treatment, increase the drug concentration in the skin, enhance patient compliance, and restrict the residual photosensitivity only to the site of application.

As to the ability of invasomes to enhance the penetration of mTHPC, the results revealed their superiority, especially of invasomes with 1 % w/v terpene mixture compared to other formulations (Fig. 5.2) (Dragicevic-Curic et al., 2008a). Invasomes with 1 % w/v terpene mixture delivered an about 3.5-, 2.7-, 2-, and 1.7-fold higher mTHPC amount to the SC than conventional liposomes, liposomes containing 3.3 % w/v ethanol, ethanolic solution of mTHPC, and invasomes containing 0.5 % w/v terpenes, respectively. Invasomes with 1 % w/v terpene mixture delivered mTHPC also into the deeper skin layers (viable epidermis and dermis). However, as to the deeper skin layers, they were only superior to the ethanolic solution of mTHPC. It is assumed that the mTHPC amounts delivered to the SC and the deeper skin layers by all formulations, except the mTHPC ethanolic solution, may be sufficient for an effective PDT (according to unpublished data from biolitec AG, Germany). The highest total penetration-enhancing effect was ascribed to mTHPC-loaded invasomes containing the highest amount of terpenes, i.e., 1 % w/v standard terpene mixture. This was in accordance with the previous study with CsA-loaded invasomes (Verma 2002).

In order to increase further the penetration-enhancing ability of invasomes containing 1 % w/v terpene mixture regarding the skin delivery of mTHPC, the ratio between d-limonene, citral, and cineole was varied in the standard terpene mixture and also single terpenes were used as additives (Dragicevic-Curic et al. 2009). As a result, seven new mTHPC-loaded invasome dispersions were obtained, which were investigated for their penetration-enhancing ability.

Obtained results revealed that, dependent on the added terpene or terpene mixture, invasomes may enhance or retard the drug penetration into the skin compared to liposomes without terpenes. The addition of 1 % w/v citral, 1 % w/v cineole, or 1 % w/v standard terpene mixture to liposomes (containing also 3.3 % w/v ethanol) resulted in the formation of highly effective skin delivery systems for mTHPC. Among these three formulations, invasomes with 1 % w/v citral provided

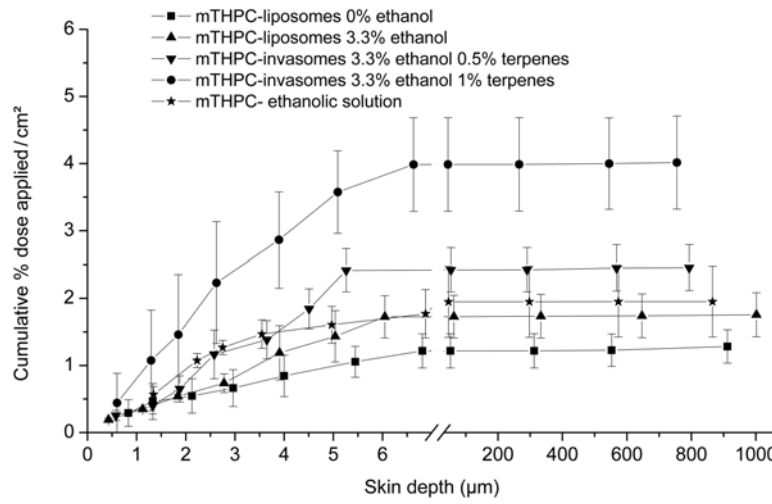

Fig. 5.2 Skin depth profile of mTHPC after 6 h nonocclusive application of different formulations onto human abdominal skin (expressed as cumulative % of dose applied/cm^2 ± SE, $n = 3$). Standard terpene mixture was used (Dragicevic-Curic et al. 2008a)

the highest overall mTHPC amount in the skin, while invasomes with 1 % w/v cineole provided a smaller total mTHPC amount in the skin than invasomes with 1 % w/v citral, but delivered, besides the high mTHPC amount in the SC, also a high mTHPC amount in the deeper skin layers, representing therefore the optimal formulation. Invasomes with 1 % w/v standard terpene mixture provided high mTHPC amounts in the SC and the deeper skin layers, but lower than invasomes with 1 % w/v cineole. Thus, these three mTHPC-loaded invasome systems might be promising for the topical PDT of cutaneous disorders.

An interesting finding in this study was that invasomes containing high amounts of d-limonene in the terpene mixture or only d-limonene exhibited low enhancement ratios, which was unexpected and difficult to explain. Limonene as a lipophilic terpene enhanced the skin delivery of the following lipophilic drugs: estradiol (Williams and Barry 1991b), indomethacin (Ogiso et al. 1995), hydrocortisone (El-Kattan et al. 2001), midazolam (Ota et al. 2003), and others. The structure–activity relationship of terpenes was also confirmed by Hori et al. (1991) and Moghimi et al. (1997), who found that hydrophilic terpenes (alcohols, ketones, and oxide terpenes, like fenchone and thymol) are more effective in enhancing the permeation of hydrophilic drugs (propranolol), while hydrocarbon terpenes (like limonene and cymene) are more effective in improving the permeation of lipophilic drugs (diazepam). The high lipophilicity of terpenes was thought to be an important property for enhancing the permeation of lipophilic drugs (Ghafourian et al. 2004). However, the addition of d-limonene failed to induce a high transport of mTHPC across the skin. This could be explained by the fact that mTHPC cannot be considered as having a high affinity to the invasomal phospholipid membrane or the skin lipids (membrane-philic or highly lipophilic), but rather as a highly hydrophobic drug.

In contrast, the highest enhancement ratio of invasomes with 1 % w/v citral was not surprising, since citral is lipophilic and should therefore enhance the skin delivery of lipophilic substances, according to Williams and Barry (1991b), Hori et al. (1991), and Moghimi et al. (1997).

As to invasomes with 1 % w/v cineole, the obtained results showing such high mTHPC penetration into the SC and deeper skin layers were unexpected, since as aforementioned, some studies showed that hydrophilic terpenes are less effective in enhancing the permeation of lipophilic drugs and were even ineffective in enhancing the permeation of the lipophilic drug indomethacin (Nagai et al. 1989; Okabe et al. 1989). However, El-Kattan et al. (2001) showed that cineole provided among 12 different terpenes the highest amount of the lipophilic hydrocortisone in the skin, which would agree with the results obtained in this study. In addition, El-Kattan et al. (2001) reported that there was no correlation between the lipophilicity of terpenes and the amount of hydrocortisone in the skin. The study with different terpene mixtures showed that besides the standard terpene mixture, also other terpene mixtures or single terpenes can be used to formulate invasomes possessing high penetration-enhancing ability.

Invasomes were shown to be able to enhance the penetration also of hydrophilic substances into the skin. Namely, invasomes and core-multishell (CMS) nanotransporters were compared regarding their ability to enhance the skin delivery of the spin label 2,2,5,5-tetramethyl-1-pyrrolidinyl-oxy-3-carboxylic acid (PCA) (Haag et al. 2011). The study has shown that CMS nanotransporters provided higher amounts of the agent in the upper layers of the SC, whereas invasomes delivered the agent into the deeper SC layers. Moreover, compared to the solution of PCA, CMS nanotransporters delivered a 2.5-fold, while invasomes delivered 1.9-fold higher PCA amount to the skin.

Moreover, Chen et al. (2011) reported that invasomes and ethosomes, regardless if they were applied in finite or infinite doses, compared to non-vesicular systems, can significantly improve the delivery of the hydrophilic dye carboxyfluorescein (CF) into the deep skin layers or across the skin. In contrast, the authors showed that in the case of mTHPC applied in finite or infinite

dose, most of the drug was accumulated in the superficial skin layers, for both vesicular systems and non-vesicular systems.

As to hydrophilic drugs, it was shown among different vesicles that conventional liposomes enhanced calcein flux 1.2 times, Transfersomes® about 1.8 times, and invasomes 7.2 times compared to the calcein aqueous solution (Ntimenou et al. 2012). Permeation (drug flux) and elasticity values for vesicles were correlated by rank order, but not linearly, indicating that elasticity can be used only as a crude predictive tool to evaluate the potential of vesicles to enhance the penetration of the hydrophilic drug through the skin. Hence, other vesicle-related properties besides elasticity may also influence the penetration-enhancing ability of vesicles. Since the cumulative calcein amount (calcein permeated up to 10 h) provided by vesicles was not significantly different among the different elastic vesicles investigated, the authors assumed that it was possible for penetration enhancers to diffuse out from invasomes and permeate through the skin lipids to enhance drug transport. Thus, besides vesicle elasticity, also other factors are most probably influencing the transport of hydrophilic drug molecules through the skin (Ntimenou et al. 2012). Drug encapsulation efficiency was not found to be an important factor influencing drug penetration into the skin.

Further, *in vitro* skin permeation and skin deposition studies demonstrated that the permeation profile of the amphiphilic drug ferulic acid through human stratum corneum epidermis membrane and the drug accumulation in the skin were both improved significantly using different liposomal systems, i.e., invasomes, polysorbate 80-based liposomes, conventional liposomes, and ethosomes compared to PBS solution of ferulic acid (Chen et al. 2010). Among all vesicles, ethosomes (with higher drug content) provided the highest skin flux and deposition of ferulic acid in the skin being 75 times and 7.3 times higher than those obtained with saturated PBS (pH 7.4) solution, respectively.

5.3.3 Penetration-Enhancing Ability of Invasomes Combined with Physical Penetration-Enhancing Methods

Invasomes were shown to be more effective in delivering hydrophilic compounds CF and radiolabeled mannitol into and through human skin *in vitro* compared to the aqueous drug solutions. For this purpose, invasomes containing 1 % standard terpene mixture were used. These invasomes were also applied in combination with skin perforation using a Dermaroller® which further enhanced drug penetration and permeation. Dermarollers with three different microneedle lengths (150, 500, and 1500 μm) were used and the one with a needle length of 500 μm appeared most promising for drug delivery into deeper skin layers or through the skin (Badran et al. 2009) (Fig. 5.3).

Trauer et al. (2014) investigated ex vivo using Franz diffusion cells the influence of massage and of occlusion, on the follicular penetration depth of invasomes and conventional rigid liposomes being loaded with a hydrophilic and a lipophilic dye. Massage, as a physical penetration-enhancing method, increased significantly follicular penetration of both conventional liposomes and invasomes while occlusion increased follicular penetration depth only of rigid liposomes. The finding that invasomes did not penetrate more effectively if occlusion was applied was expected as for deformable liposomes, transepidermal water gradient is needed for an efficient skin penetration (Cevc et al. 1998). The results confirmed that massage is a potent tool for increasing follicular penetration of vesicles.

5.3.4 *In vivo* and *In Vitro* Therapeutic Effectiveness of Invasomes

In order to prove the therapeutic effectiveness of CsA-loaded invasomes, Verma et al. (2004) performed a study using 0.5 % CsA invasomes containing 2 % w/v standard terpene mixture in

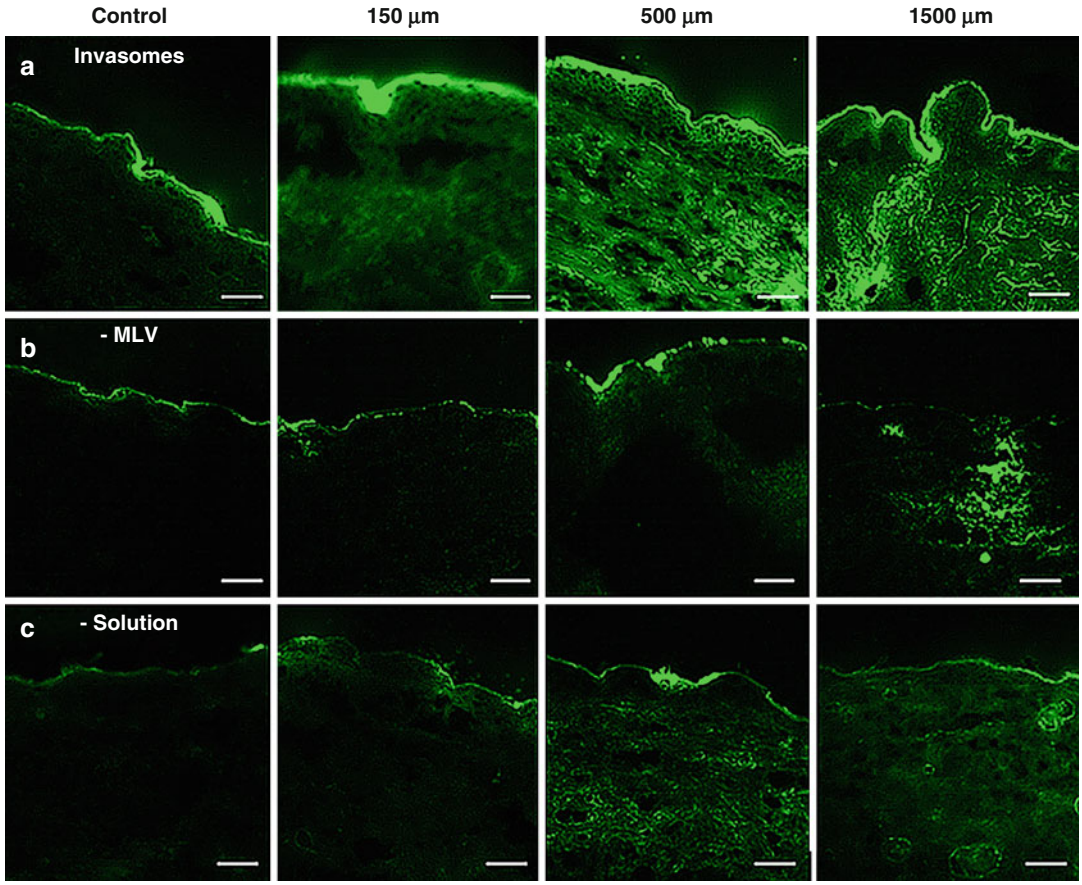

Fig. 5.3 Fluorescence microscopic images of the cross section of human abdominal skin after 6 h of incubation with different formulations of the fluorescent dye CF ((**a**) invasomes, (**b**) non-extruded multilamellar invasomes (MLV), and (**c**) buffer solution) and pretreatment with the Dermaroller® of different microneedle length. Scale bars represent 100 µm (Badran et al. 2009)

the treatment of alopecia areata in the Dundee Experimental Bald Rat (DEBR) model. CsA-loaded invasomes were compared to conventional liquid-state CsA-loaded liposomes and to the CsA ethanolic solution. Hence, 15 rats were divided into three groups. All rats were treated with CsA twice a day for 6 weeks within a bald flank, while the contralateral flank received an equivalent control formulation. The obtained results suggested that CsA liposomes with (i.e., invasomes) and without terpenes have promising potential as a topical treatment for alopecia areata, showing hair regrowth, reduced inflammatory infiltrate, and improved hair follicle morphology on treated sites. Invasomes induced a faster visible hair regrowth on the drug-treated site than conventional liposomes (see Fig. 5.4 for the effect of invasomes). In contrast, the ethanolic solution of CsA showed neither visible signs of hair growth nor reduction of hair follicle inflammation. The results indicated that CsA in liposomes provided a localized hair growth-promoting effect, while the synergistic effect of ethanol, terpenes, and phospholipids might be an explanation for the enhanced delivery of CsA by invasomes and any accumulation of the vesicles in the hair follicle (Verma et al. 2004).

Two mTHPC invasome dispersions, invasomes with 1 % w/v citral and invasomes with 1 % w/v terpene mixture, and the ethanolic mTHPC solution were used for PDT of mice bearing the subcutaneously (s.c.) implanted

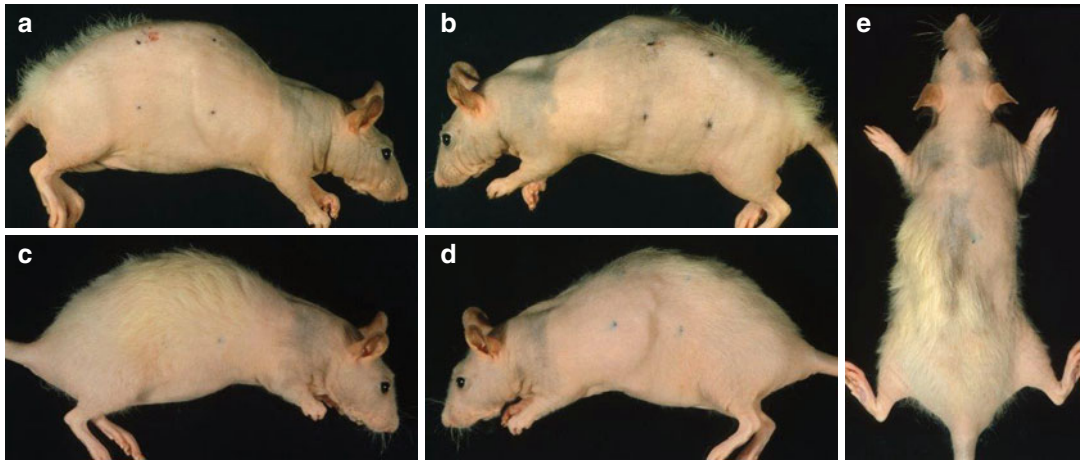

Fig. 5.4 Hair growth before and after treatment of DEBR with CsA invasomes. At the start of therapy, complete loss of hair on the flanks can be seen within and beyond the marked area, as well as hair loss on head and shoulders (**a**, **b**). After 6 weeks of topical application of CsA invasomes, the treated area is fully rehaired with some hair fibers longer than would be expected with a normal rat pelage (**c**). Control-treated skin did not show any growth of hair (**d**) as compared to the drug-treated skin (**c**, **e**) (Verma and Fahr 2004)

human colorectal carcinoma HT29 (Dragicevic-Curic et al. 2008b). This pilot PDT study was performed with the aim to test whether the chosen mTHPC-loaded invasomes can reduce tumor size by PDT or at least slow down tumor growth compared to the control group (mice without any treatment). mTHPC invasomes containing 1 % w/v terpene mixture, showing the best results, were not able to reduce the tumor size. However, their application induced a slower tumor growth compared to the control despite the high invasivity, intermediate sensitivity to PDT, and subcutaneous localization of the HT29 carcinoma, which limited the success of PDT. Thus, these results, despite not showing a tumor decrease, are promising and indicate that mTHPC invasomes might be a good modality for the PDT treatment of skin disorders which are more sensitive to PDT, less invasive, and more accessible, like psoriasis or different superficial skin tumors (Bowen's disease, basal cell carcinoma).

These mTHPC-loaded invasomes and the ethanolic solution, which were used in the PDT of mice bearing the s.c. implanted tumor HT29 (Dragicevic-Curic et al. 2008b), were further investigated for their photodynamic efficacy *in vitro* in two tumor cell lines, i.e., in the human epidermoid carcinoma cell line A431 and the human colorectal carcinoma cell line HT29 (Dragicevic-Curic et al. 2010). The results revealed that invasomes and the ethanolic solution used at a 2 μM mTHPC concentration and photoirradiation at 20 J/cm^2 were able to reduce survival of HT29 cells and especially of A431 cells, being more sensitive to PDT. In contrast to HT29 cells, where there was not a significant difference between cytotoxicity of the mTHPC ethanolic solution and mTHPC invasomes, in A431 cells, mTHPC invasomes were more cytotoxic (Fig. 5.5). Survival of about 16 % of A431 cells treated with invasomes is very promising, since it demonstrates invasomes' potential to be used in topical PDT of cutaneous malignant diseases.

5.3.5 Fluidity of Invasomes

Among all properties, the fluidity, i.e., thermodynamic state of liposomes' bilayers, influences at most their penetration-enhancing ability. As liquid-state (fluid) vesicles have been found to be superior over gel-state (rigid) vesicles in terms of increasing the drug penetration into the skin (van Kuijk-Meuwissen et al. 1998a, b; El Maghraby et al. 1999, 2001a, b), it was assumed that invasomes, showing high penetration-enhancing

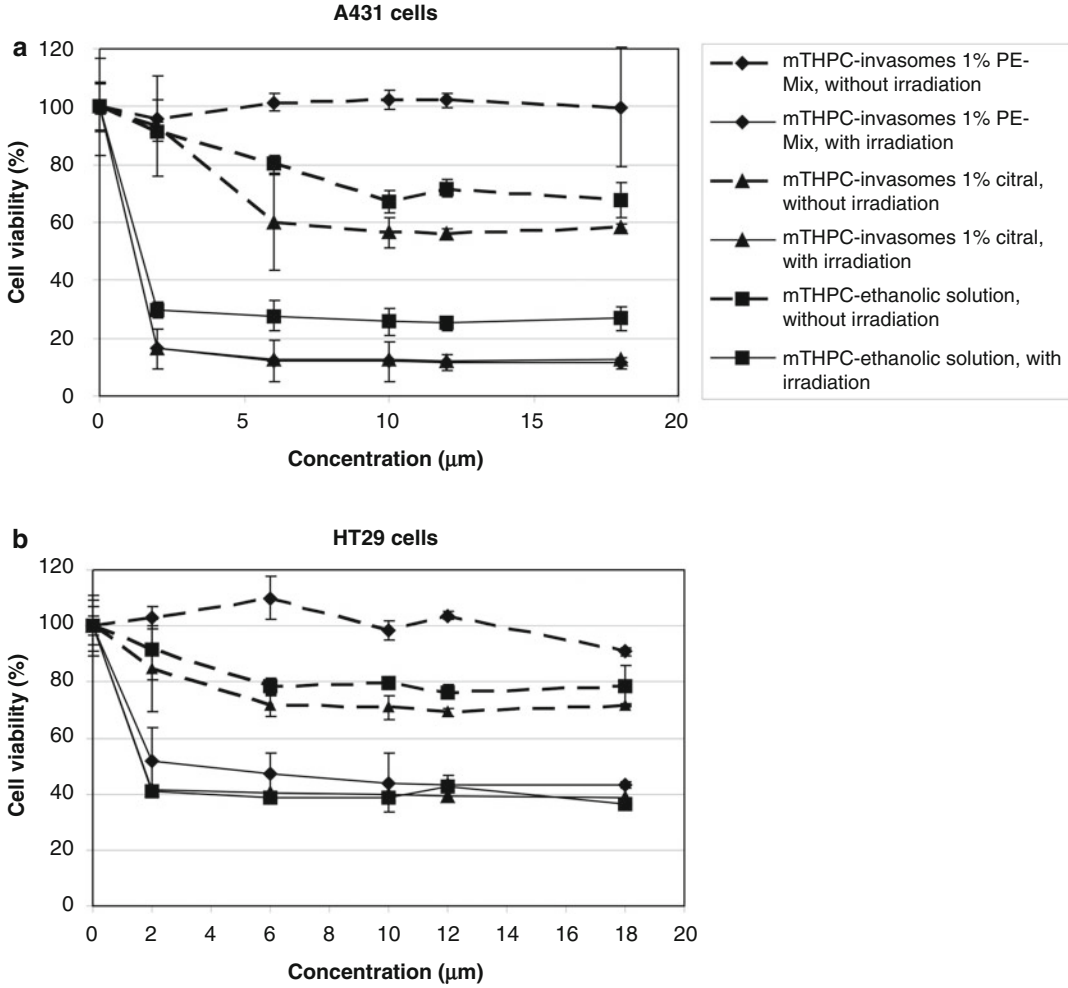

Fig. 5.5 Dark and photocytotoxicity of three mTHPC formulations used at different mTHPC concentrations against A431 and HT29 tumor cells after 24 h of incubation. For the photocytotoxicity study, tumor cells were photoirradiated with a light dose of 20 J/cm^2. (**a**) A431 cells and (**b**) HT29 cells. *PE* penetration enhancer = standard terpene mixture (Dragicevic-Curic et al. 2010)

ability (Dragicevic-Curic et al. 2008a, 2009), possess high membrane fluidity.

The results obtained by electron spin resonance (ESR) measurements revealed that the addition of 1 % w/v of a single terpene or terpene mixture to liposomes containing 3.3 % w/v ethanol in order to obtain invasomes increased significantly the phospholipid fluidity near to the C16 atom of their acyl chains, compared to liposomes without terpenes (conventional liposomes and liposomes containing 3.3 % w/v ethanol) (Dragicevic-Curic et al. 2011). Thus, ESR showed that invasomes indeed represent vesicles of higher membrane fluidity than conventional liposomes. However, the membrane fluidity did not differ markedly among different invasomes. It was not possible to differentiate between the influences of each single terpene/terpene mixture on the invasome fluidity, and hence, there was also no direct correlation between the invasomes fluidity and their penetration-enhancing ability (Dragicevic-Curic et al. 2008a, 2009). However, conventional liposomes (being in fluid thermodynamic state, but significantly lower than invasomes) provided the second lowest mTHPC amount in the

skin compared to different invasomes (after invasomes with the terpene mixture containing the highest amount of d-limonene). According to ESR data, the addition of all terpenes/terpene mixtures increased the vesicle fluidity, while not all terpenes/terpene mixtures increased the penetration-enhancing ability of invasomes to improve the skin delivery of mTHPC compared to conventional liposomes or liposomes containing 3.3 % ethanol (Dragicevic-Curic et al. 2009). The ESR results are in agreement with differential scanning calorimetry (DSC), which also showed that the addition of 1 % w/v terpenes/terpene mixtures increased the molecular motional freedom of phospholipid acyl chains in invasome bilayers (Dragicevic-Curic et al. 2011). The ESR and DSC results are also in agreement with the cryoelectron microscopy investigation (Dragicevic-Curic et al. 2008a), which showed that the addition of terpenes had an influence on the shape of vesicles, i.e., besides spherical vesicles, deformed vesicles of different shapes were also present in invasome dispersions and an increase of the terpenes' amount resulted in their increased number (Fig. 5.6). It was assumed that the addition of terpenes, especially 1 % w/v terpenes, to already liquid-state (fluid) liposomes with 3.3 % w/v ethanol increased further their membrane fluidity. The obtained invasomes were, thus, of very high membrane fluidity compared to liposomes without terpenes, which was confirmed by cryoelectron microscopy, ESR, and DSC measurements.

Since penetration studies, ESR and DSC (Dragicevic-Curic et al., 2008a, 2009, 2011) did not show a direct correlation between fluidity and penetration-enhancing ability of invasomes, it was proposed that besides fluidity, other phenomena might also be involved in the mechanism of

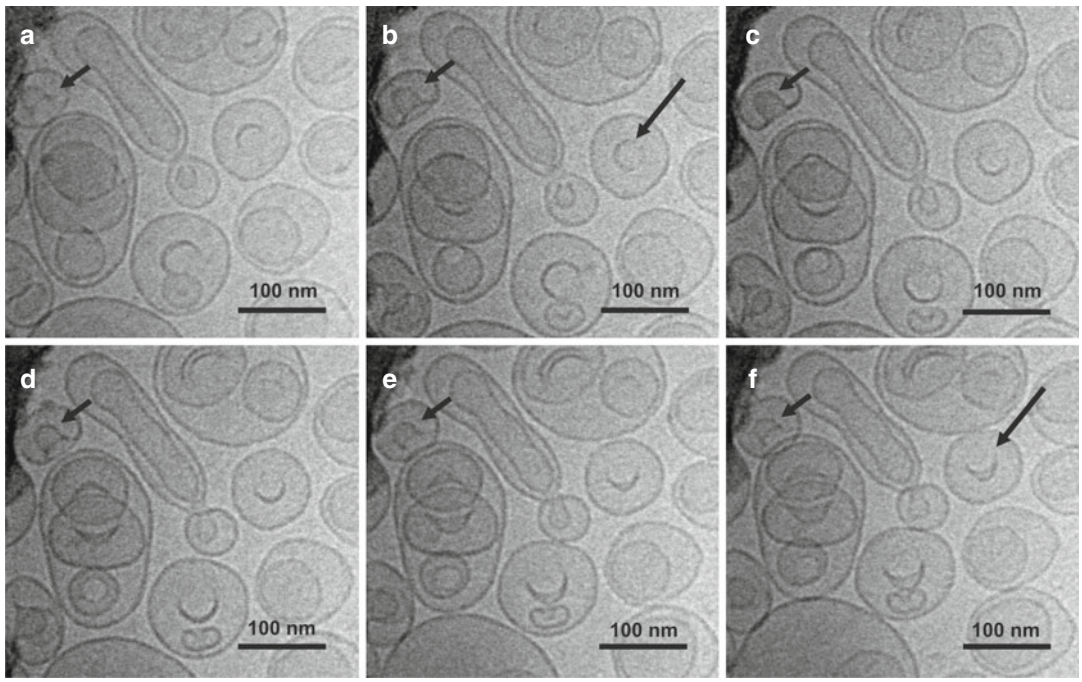

Fig. 5.6 Cryoelectron microscopy of mTHPC invasomes containing 1 % w/v terpenes (the angle of the goniometer was changed from −25° to +30°). *Arrows* in the micrographs (**a**–**f**) depict same deformed vesicles but viewed from different angles: *short arrows* represent a deformed vesicle, which shape changed its appearance from a cup-like shape (**c**) to a shape similar to bilamellar liposomes (**f**); long *arrows* represent a deformed vesicle which first looked like a bilamellar vesicle (**b**), but afterward, it was shown that the vesicle has an invagination (**f**). (Dragicevic-Curic et al. 2008a)

the skin penetration enhancement induced by invasomes. Hofland et al. (1994) proposed, for example, two mechanisms for nonionic vesicles to play an important role in the vesicle–skin interactions, leading to an enhanced drug penetration: the penetration-enhancing effect of the surfactant molecules and the effect of the vesicular structure. Therefore, in the case of invasomes, besides the effect of the fluid vesicular structure, also the penetration-enhancing effect of invasome constituents can be assumed. The constituents of invasomes, i.e., phospholipids, ethanol, and terpenes, represent potent penetration enhancers (El-Kattan et al. 2001; Yokomizo and Sagitani 1996a, b; Mutalik et al. 2009), which if released from fragmented invasomes could synergistically fluidize the intercellular lipid layers in the SC and thereby increase the skin penetration of drugs and possibly of small invasomes. However, this is only an assumption. The mechanism by which invasomes induce drug penetration enhancement should be investigated in further experiments.

5.3.6 Mode of Action of Invasomes

Analyzing the results from the aforementioned studies (Verma 2002; Verma and Fahr 2004; Dragicevic-Curic et al. 2008a, 2009, 2011), it is assumed that the reason for the superior behavior of certain invasomes compared to conventional liposomes, liposomes containing small amounts of ethanol and the ethanolic solution, is the presence of terpenes and ethanol in liposomes, i.e., the synergistic effect of ethanol, terpenes, and liposomes. Liposomes have been used for years to enhance the skin delivery of various drugs (El Maghraby and Williams 2009). Further, all constituents of invasomes, i.e., terpenes (Kunta et al. 1997; El-Kattan et al. 2001), as well as ethanol (Berner et al. 1989; Mutalik et al. 2009; Jaimes-Lizcano et al. 2010) and phospholipids (Yokomizo and Sagitani 1996a, b), have also been shown to be very potent penetration enhancers, as aforementioned. As to phospholipids, invasomes were made of PC, which, due to its head group, having a strong enhancing effect, presents a strong penetration promoter (Yokomizo and Sagitani 1996a). In addition, the acyl chains of the PC were unsaturated, which further increased the phospholipids' penetration-enhancing ability (Yokomizo and Sagitani 1996b). Moreover, synergistic effects of ethanol and terpenes (Puglia et al. 2001; Vaddi et al. 2002; Ota et al. 2003), ethanol and liposomes (Kirjavainen et al. 1999b; Touitou et al. 2000; Verma and Fahr 2004), and ethanol, terpenes, and liposomes (Verma 2002) in enhancing the drug permeation have been reported in literature.

On the basis of gained results in these studies (Verma 2002; Verma and Fahr 2004; Dragicevic-Curic et al. 2008a, 2009, 2011) and studies performed by other authors (Cevc and Blume 1992; Cevc et al. 2002; Honeywell-Nguyen et al. 2002; Verma 2002), the following mechanism of the penetration-enhancing ability of invasomes was proposed. As invasomes were in all studies applied in finite doses under nonocclusion, a number of concomitant processes could take place. Small amounts of ethanol from the invasome dispersion (being outside the vesicles) could be able to fluidize the intercellular SC lipids (Berner and Liu 1995; Barry 2001). Further, a high part of invasomes is probably fragmented in their attempt to penetrate into the upper SC layers, which leads to the release of terpenes, ethanol, and unsaturated phospholipids. This would be in agreement with findings of most authors (Hofland et al. 1994; Zellmer et al. 1995; Kirjavainen et al. 1996, 1999b), who propose that vesicles disintegrate at the skin surface and that vesicle components (i.e., phospholipids) penetrate molecularly dispersed into the intercellular lipid matrix, where they mix with the intercellular lipids of the SC thereby modifying the lipid layers and leading to an enhanced drug penetration. Thus, the released ethanol, terpenes, and unsaturated phospholipids would be free to exert their penetration-enhancing effect. It is proposed that these penetration enhancers would synergistically act on fluidizing the intercellular SC lipids, since all of them act via this mechanism (Berner and Liu 1995; Kirjavainen et al. 1999a; Thakur et al. 2006). This could lead to the formation of microcavities and to an increase of the free volume for drug diffusion (Barry

2001), which could further increase the diffusion coefficient of the drugs released from vesicles. In addition, also the partitioning of the drug into the intercellular lipid bilayers of the SC would be increased by phospholipids (Kirjavainen et al. 1999a), ethanol (Megrab et al. 1995), and terpenes (Williams and Barry 1991b). However, since the constituents of invasomes, being potent penetration enhancers, act also via other mechanisms (see in the book series Percutaneous Penetration Enhancers, the volume Chemical Methods in Penetration Enhancement, Modification of the Stratum Corneum), these mechanisms could also have an influence on the enhanced penetration of the drug. Regarding the penetration of vesicles, a lot of phenomena are included which synergistically might facilitate the penetration of some small intact invasomes into the SC, such as: (1) disturbed organization of the SC lipids, (2) high fluidity of invasomes due to the effect of terpenes and ethanol (confirmed by Dragicevic-Curic et al. 2011), (3) probably high deformability of invasomes (assumed due to the correlation between vesicles' high fluidity and deformability) (Godin and Touitou 2003), (4) small particle size of vesicles, and (5) presence of the transepidermal osmotic gradient, which is an important driving force for the diffusion of intact deformable vesicles of high hydrophilicity as they tend to follow the hydration gradient across the skin (Cevc and Blume 1992). The release of the drug in the skin layers could be a result of fusion of penetrated vesicles with the intercellular lipids of the SC (Touitou et al. 2000). Verma (2002) proposed the penetration of small invasomes through SC bilayers, disturbed due to the effect of ethanol and terpenes on the SC. Further, according to Verma (2002), the pilosebaceous units appeared to be a major route of invasomes' penetration into the skin. The penetration of intact deformable vesicles through the skin was proposed by Cevc et al. (2002, 2003). Honeywell-Nguyen et al. (2002) proposed also the penetration of intact elastic vesicles through channel like regions into the deeper layers of the SC. Touitou et al. (2000) assumed that ethosomes could penetrate into the SC bilayers, being disturbed due to the effect of ethanol. However, the penetration of intact vesicles is rejected by most authors (Lasch et al. 1991; Hofland et al. 1994; Zellmer et al. 1995; Kirjavainen et al. 1996, 1999a).

Conclusion

Invasomes have proven to be an efficient skin delivery system for lipophilic as well as even to a higher degree for hydrophilic drugs. Different single terpenes or terpene mixtures can be used to formulate invasomes. Dependent on the added terpene or terpene mixture, invasomes may enhance or retard the drug penetration into the skin compared to liposomes without terpenes. Thus, besides described terpene mixtures, other mixtures should be investigated, i.e., the composition of invasomes could be further improved, by exploring the possibility of using other terpenes and terpene mixtures as constituents of invasomes. This could lead to the development of novel invasomes being more efficient skin delivery systems than existing invasomes.

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6 Penetration Enhancer-Containing Vesicles for Cutaneous Drug Delivery

Maria Manconi, Chiara Sinico, and Anna Maria Fadda

Contents

Abbreviations

CLSM	Confocal laser scanning microscopy
D	Dialyzed
DCF	Diclofenac
DCF_{Na}	Diclofenac sodium salt
DDS	Drug delivery systems
E%	Entrapment efficiency
FTIR	Fourier transform infrared spectroscopy
HLB	Hydrophile-lipophile balance
Lab	Labrasol® (caprylocaproyl macrogol-8 glyceride, Gattefossé, France)
MLVs	Multilamellar vesicles
ND	Non-dialyzed
OA	Oleic acid
Or	Decylpolyglucoside (Oramix™ NS10, Seppic, France)
P50	Phospholipon 50 (Lipoid GmbH, Germany)
P90G	Phospholipon 90G (Lipoid GmbH, Germany)
P90H	Phospholipon 90H (Lipoid GmbH, Germany)
PBS	Phosphate-buffered saline
PC	Phosphatidylcholine
PDI	Polydispersity index
PE	Penetration enhancer
PEG 400	Polyethylene glycol 400
PEV-1	Vesicles with Labrasol®
PEV-2	Vesicles with Transcutol®
PEV-3	Vesicles with cineole
PEVs	Penetration enhancer-containing vesicles
PG	Propylene glycol
SC	Stratum corneum
SEM	Scanning electron microscopy
SUV	Small unilamellar vesicles
Tc	Transition temperature
TEM	Transmission electron microscopy
TRA	*Trans* retinoic acid
Trc	Transcutol® (diethylene glycol monoethyl ether, Gattefossé, France)

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6.1 Introduction

The skin can offer several advantages as a route of drug administration although its barrier nature makes it difficult for most drugs to penetrate into and permeate through it.

During the last decades there has been a large interest in nanocarriers as a tool to improve skin delivery of drugs. Among the several nanosystems, vesicles have been largely studied, although their function as skin drug delivery systems (DDS) is controversial with variable effects being reported in relation to the type of vesicles and their composition. In fact, vesicles can act as drug carriers controlling the active release; they can provide a localized depot in the skin for dermally active compounds; they may also afford targeted delivery to skin appendages. Moreover, vesicles can enhance transdermal drug delivery, increasing systemic drug concentration (Sinico and Fadda 2009).

Although some authors suggested that liposomes could be suitable candidates for transdermal delivery, most studies have shown that conventional liposomes fail to penetrate the skin layers deeply (Choi and Maibach 2005; Gupta et al. 2012). Therefore, it is generally accepted that conventional liposomes act as "skin drug localizers" and hence as very suitable drug carriers for skin diseases. Furthermore, it is also recognized that thermodynamic state of the lipid bilayers (liquid or gel state) is fundamental in determining liposome efficacy in topical drug delivery, showing that gel-state vesicles are less effective in increasing drug permeation through the skin than liquid-state vesicles (Hofland et al. 1994; Fahr and Chen 2010). However, other physicochemical properties of the vesicles such as size, lamellarity, and charge affect also their effectiveness as skin DDS (Du Plessis et al. 1994; Verma et al. 2003; Sinico and Fadda 2009).

The lack of ability of conventional liposomes to efficiently deliver drugs across the skin has led to intensive research with the introduction and development of new classes of lipid vesicles. In 1992, Cevc and Blume introduced the first generation of the highly deformable, elastic liposomes (Transfersomes®; see Chap. 3), Touitou et al. (1997) developed ethosomes (see Chap. 5), and new elastic nonionic surfactant vesicles were introduced by Van den Bergh et al. (1999). Able to enhance transdermal drug delivery, they are also reported to improve skin deposition of drugs with a higher efficacy than conventional liposomes (El Maghraby et al. 2000; Trotta et al. 2002).

In addition, a variety of penetration enhancer (PE) molecules have been tested as "edge activators" in the formulation of new deformable vesicles by different authors. In particular, El Maghraby et al. (2000) incorporated oleic acid (OA) into the lipid bilayer obtaining deformable vesicles capable of improving transepidermal estradiol flux. Dragicevic-Curic et al. (2008, 2009) used a mixture of terpenes (0.5–1 %) and ethanol (3 %) to produce the new "invasomes" that showed the capability to deliver temoporfin to the stratum corneum (SC) and deeper skin layers (see Chap. 6). Propylene glycol has also been suggested in new vesicular formulations as an alternative to ethanol (Elsayed et al. 2007).

In this context, our group has been studying for years the so-called penetration enhancer-containing vesicles (PEVs), liposomes containing in their composition a PE, as carriers for dermal delivery of different drugs (Manconi et al. 2009, 2011a, b, 2012; Mura et al. 2009; 2011; 2012; Chessa et al. 2011; Caddeo et al. 2012, 2013). During these years, PEVs have been prepared using various PEs, of different physicochemical properties and mechanism of enhancement, with the aim of finding new stable and efficient vesicular carriers for dermal and transdermal drug delivery. Moreover, a particular attention has been devoted to PEVs prepared by using hydromiscible cosolvents that have shown peculiar properties and mode of action.

6.2 PEVs: Development and Features

6.2.1 Composition and Preparation of PEVs

PEVs have shown to be very versatile vesicular systems than can be prepared by associating vari-

ous PEs to different phospholipids, in different amounts. Among the aqueous cosolvents, diethylene glycol monoethyl ether (Transcutol®, Gattefossé, France (Trc)), propylene glycol (PG), and glycerol were the most studied. During these years we have also been studying PEVs with hydrophilic, hydromiscible surfactants such as caprylocaproyl macrogol-8 glyceride (Labrasol®, Gattefossé, France (Lab)), polyethylene glycol 400 (PEG 400), and decylpolyglucoside (Oramix™ NS10, Seppic, France (Or)). Cineole was tested as a lipophilic PE in the preparation of PEVs, while oleic acid was used to induce a negative surface charge in some samples, and it increased the absolute value of surface charge of the vesicles.

Nevertheless, whatever the PE used in the formulation, the PEVs can be obtained by the several methods commonly used in the preparation of liposomal carriers, as shown in Table 6.1. In fact, at first, PEVs were prepared by the classic thin lipid film (Manconi et al. 2009; Caddeo et al. 2012) and then by the rehydration-hydration method (Mura et al. 2009, 2012), and successively other methods were tested. In particular, a simple mechanical shaking of the phospholipids in the PE-containing aqueous medium was proved to be an easy procedure capable of avoiding the use of organic solvents (Chessa et al. 2011; Mura et al. 2011). An improvement of the lipid film technique was reached by hydrating the film in two steps (i.e., using at first only half amount of the aqueous medium and mechanically shaking for 1 h before completing hydration with the second aliquot of the medium), followed or not by probe sonication, thus improving homogeneity of the vesicular populations (Manconi et al. 2011a, b).

One peculiarity of the studied PEVs is that, whatever the method used for their preparation (i.e., with or without sonication) and independently from the obtained vesicle structure (multi-, oligo-, or unilamellar), they have always been obtained with a mean diameter in the nanometer range and an acceptable homogeneity (polydispersity index, PDI, generally ≤ 0.3), except when the hydromiscible Trc was used in high concentrations (40–50 %) for diclofenac delivery. In this case, the obtained vesicles showed a mean size higher than 1 μm, a PDI around 1, and a high instability (Manconi et al. 2009).

Moreover, the presence of the PE was also demonstrated to affect the vesicle structure. Indeed, X-ray diffraction studies (small- and wide-angle X-ray scattering) revealed that association of Trc to a cheap commercial mixture of phosphatidylcholine (PC), rich also in free fatty acids and other lipid components (Phospholipon 50, P50, Lipoid GmbH, Germany), was able to produce small unilamellar vesicles (SUV) or multilamellar vesicles (MLVs) independently from the method (hydration in two steps followed or not by sonication) used for their preparation. The structure of obtained vesicles was affected by the used amount of the PE: 30 % Trc-PEVs were always multilamellar, while with a lower Trc-content, all vesicles were unilamellar (Manconi et al. 2012)

Results in terms of entrapment efficiency (E%) of loaded drugs were generally good (Table 6.1), but different depending on the physicochemical properties of the model drug and vesicle composition. Thus, lipophilic molecules such as tretinoin were loaded in high percentage (>80 %, Table 6.1) in all the tested formulations. Diclofenac acid (DCF) and diclofenac sodium (DCF_{Na}) were studied in PEVs prepared with the hydromiscible cosolvents Trc and PG, and E% was different as a function of the used PE, its amount, and hydrophilic or lipophilic form of the drug. In particular, DCF was incorporated in higher yields (65–75 %) than DCF_{Na} (around 50–57 %).

However, the composition of PEVs can be optimized for each drug by using different types and concentrations of phospholipids and the appropriate PE in a right amount. In particular, results have shown that generally Trc cannot be used in a concentration higher than 30 %. Only when Trc-PEVs were prepared as carriers of quercetin, small (86 ± 5 nm), stable, and with high entrapment efficiency (75 ± 9 %) vesicles were obtained even with a concentration of 40 % Trc (Chessa et al. 2011).

Another peculiarity of the PEVs is their high stability under storage, i.e., vesicle mean diame-

Table 6.1 Composition, preparation method, and properties of PEVs

Composition	Model drug	Preparation method	Vesicle structure	Mean size range (nm)	Mean Drug loading (%)	Findings	Ref.
Soy PC, transcutol (Trc), propylene glycol (PG)	Diclofenac	Thin lipid film	MLV	300–350 (PG)	30–65 (Trc)	High vesicle stability	Manconi et al. (2009)
				300–450 (Trc up 30 %)			
				1780–4000 (40–50 % Trc)	38–60 (PG)		
Soy PC, dicetylphosphate, labrasol, Trc, cineole	Minoxidil	Dehydration-rehydration	DRV	202 (labrasol)	59–71	High drug dermal delivery with the most deformable PEVs (labrasol and cineole)	Mura et al. (2009), Mura et al. (2012)
				171 (transcutol)			
				144 (cineole)			
Soy PC, Trc	Minoxidil	Mechanical shake	MLV	165 (5 % Trc)	67–68	Trc-PEVs do not significantly enhance minoxidil delivery to the skin	Mura et al. (2011)
				199 (10 % Trc)			
				200 (20 % Trc)			
				320 (30 % Trc)			
Enriched soy PC (P90), oleic acid, Trc	Diclofenac acid, Diclofenac sodium salt	Hydration in two steps of the thin lipid film + sonication	SUV	98–132 (DCF)	65–75 (DCF)	Superior ability of Trc-PEVs to enhance DCF and DCF_{Na} delivery into and through the skin. PEVs act as true carriers	Manconi et al. (2011a)
				87–114 (DCF_{Na})	50–57 (DCF_{Na})		
Soy PC (P50), Trc, decylpolyglucoside (Or), labrasol, PG	Tretinoin	Hydration in two steps of the thin lipid film	MLV	133 (Or)	91	Improved cutaneous retention and reduced transdermal delivery. Evidence of PEVs' interaction with intercellular lipids	Manconi et al. (2011b)
				137 (labrasol)	84		
				164 (Trc)	82		
				157 (PG)	86		
Soy PC (P50), Trc, labrasol, PG, PEG 400	Quercetin	Mechanical shake followed by sonication	MLV	83 (PG)	57	PEs make quercetin to be loaded into vesicles. Drug skin accumulation is affected by the used PE	Chessa et al. (2011)
				190 (PEG400)	48		
				86 (labrasol)	75		
				226 (transcutol)	59		
Soy PC (P50), Trc	Diclofenac sodium salt	(a) Hydration in two steps of the thin lipid film (b) As above but followed by sonication	SUV MLV (30 % Trc)	(a) 168–182 423 (30 % Trc, shaken) (b) 96–100 172 (30 % Trc)	(a) 50–57 (b) 39–51	Influence of lipid composition on PEVs features. Synergic effect of Trc that improves PEVs' bilayer fluidity and perturbs intercellular lipids	Manconi et al. (2012)

Soy phosphatidylcholine (P50), oleic acid, labrasol	Minoxidil	(a) Thin lipid film[a]	(a) MLV	(a) 154–199 (labrasol in lipid phase) 125–199 (labrasol in aqueous phase)	(a) 63–76 (labrasol in lipid phase) 68–86 (labrasol in aqueous phase)	Labrasol is more efficient in enhancing minoxidil cutaneous delivery when added to the lipid phase in small amounts	Caddeo et al. (2012)
		(b) Thin lipid film + sonication[a]	(b) OLV	(b) 106–122 (labrasol in lipid phase) 101–125 (labrasol in aqueous phase)	(b) 61–66 (labrasol in lipid phase) 62–74 (labrasol in aqueous phase)		

[a]Labrasol either in the aqueous or in the lipid phase

ter and zeta potential values were constant during at least 3 months at 4 ± 1 °C (Mura et al. 2011; Manconi et al. 2011a; Manconi et al. 2012).

6.2.2 Characterization of PEVs

PEVs have been characterized by using different methods to obtain more information regarding their properties and the role of the used PE in affecting these properties as well as the skin delivery of drugs. Transmission electron microscopy (TEM) and optical or polarized light microscopy gave evidence of vesicle formation and morphology, and dynamic and electrophoretic light scattering (Zetasizer nano-ZS, Malvern Instruments, Worcestershire, UK) was used for size distribution and zeta potential determination. Rheological measurements were employed to study viscoelastic properties of the vesicles and thermodynamic state of the lipid bilayers (Manconi et al. 2011a). Since it is well known that vesicle ability to squeeze through skin pores depends on the deformability of the vesicular bilayers, we evaluated the aptitude of the different PEs to produce elastic vesicles, using soy PC as the phospholipids. The deformability of the vesicular membrane was evaluated by extruding the vesicle dispersions through polycarbonate filters with pores smaller than vesicles' mean size (50 nm). X-ray diffraction (small- and wide-angle X-ray scattering) was employed to ascertain the vesicle structure and to give information on the effect of the vesicles' composition on their structure.

At first, PEVs were studied as carriers of therapeutic amount of DCF, by using increasing quantities of PG and Trc, being two hydromiscible glycol moiety-containing cosolvents (Manconi et al. 2009). While PG-liposomes were already shown by Elsayed et al. (2007) to improve in vivo skin deposition of cinchocaine in comparison with other vesicular formulations (traditional liposomes, ethosomes, and deformable liposomes), in this study Trc, which is a well-known and safe PE, was for the first time used as component of liposomes (Manconi et al. 2009). Both empty and DCF-loaded PEVs were prepared proving that liposomes can be obtained by using increasing amounts of the two PEs up to 50 % v/v in phosphate-buffered saline (PBS) of pH 7.0. A high concentration of soy PC (180 mg/ml) was needed to load high amounts of the model drug DCF (10 mg/ml). Results showed that liposomal structure can be obtained with both hydromiscible permeation enhancers, although the highest Trc concentrations (40–50 %) led to very large and unstable aggregates (Table 6.1). As to the E%, obtained vesicles showed a mean loading capability that increased as the PG concentration increased, while when Trc was used, the E% was high only in 10 and 20 % Trc-containing vesicles, being even higher than in the corresponding PG-vesicles (Fig. 6.1).

The main result of this research was the proof that the vesicle formation was easier in the presence of both PEs, as demonstrated by the rheological behavior. In fact, vesicle formation was studied starting from lamellar phases obtained by destroying vesicles (freeze and thaw technique). The energy required to reform the vesicles was measured by means of shear stress vs. shear rate curves (by increasing and then reducing the applied shear stress for six times) and thus obtaining a hysteresis loop area. Figure 6.2 shows that PG and Trc facilitated vesicle formation from the lamellae allowing a reduction of the hysteresis loop area as well as the apparent viscosity (Manconi et al. 2009). Moreover, the 10–40 % PG- and 10–20 % Trc-containing liposomes showed high stability during storage for 28 days at 4 °C.

6.2.3 Ex Vivo Skin Penetration/ Permeation Studies

Ex vivo (trans)dermal drug delivery experiments were always carried out using Franz vertical diffusion cells and newborn pig skin under non-occlusive conditions, since they are known to be detrimental to drug accumulation into the skin (Bouwstra et al. 2003). Moreover, non-occlusive conditions are supposed to improve patient compliance. Conventional liposomes, having the same composition of the corresponding PEVs but

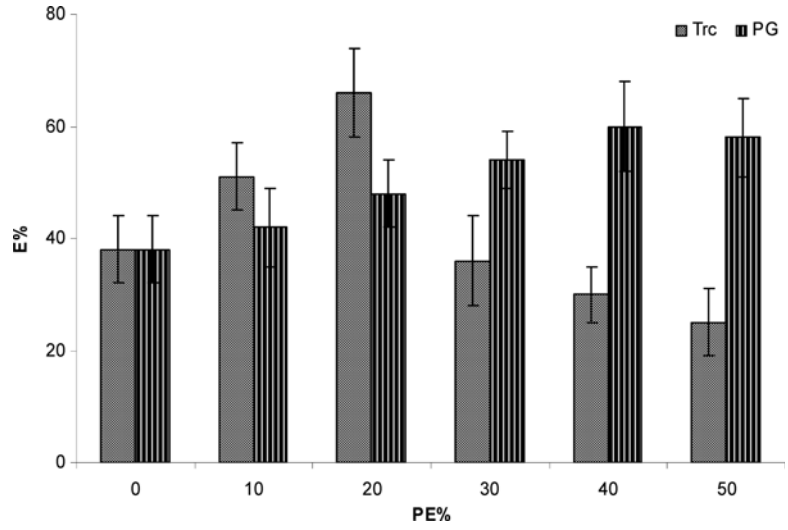

Fig. 6.1 Influence of glycol concentration on incorporation efficiency (E%) of diclofenac (From Manconi et al. 2009)

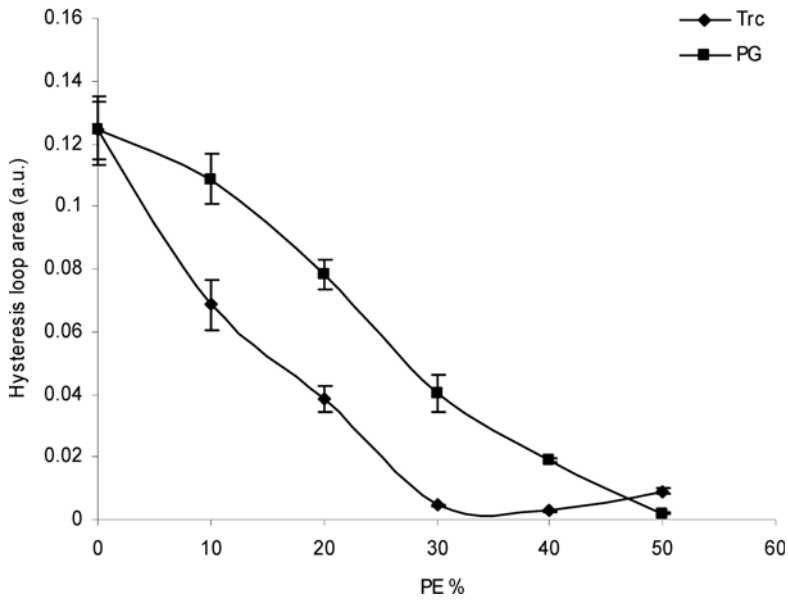

Fig. 6.2 Hysteresis loop area of soy lecithin dispersions vs. percentage of permeation enhancer in vesicle formulations. The error bars in the hysteresis loop area values are SD of three independent samples (From Manconi et al. 2009)

without the PE, were generally used as a control. When performed, pretreatment experiments were carried out by treating the pig skin with empty vesicles for 8 h in Franz cells and then by applying the appropriate drug solution. Confocal laser scanning microscopy (CLSM) and scanning electron microscopy (SEM) were used to study PEVs' effect on the skin layers.

A review of the results obtained in our studies with different model drugs and PEVs is as follows.

6.2.3.1 Minoxidil

In the first study aimed at improving cutaneous drug delivery by PEVs, the new vesicles were tested as carriers of minoxidil, one of the most widely used drugs for the treatment of male and female hair loss. Here, Trc-PEVs were compared with PEVs prepared with the lipophilic PE cineole and the hydromiscible Lab, which is very different in structure from Trc. In fact, Lab is a commercial nonionic hydrophilic surfactant (HLB = 14) composed of a well-defined mixture

of mono-, di-, and triglycerides and mono- and di-fatty acid esters of polyethylene glycol. All the PEs were tested in the same amount (2 %) (Mura et al. 2009).

Deformability studies evidenced that Trc-PEVs were only more deformable than conventional liposomes (control), while PEVs prepared with Lab and cineole were, respectively, six- and fivefold more elastic than control and three- and twofold more deformable than Trc-PEVs (Table 6.2).

Therefore, different deformability of vesicles obtained with the two hydrophilic PEs was the consequence of the different structures of Lab and Trc. In fact, the former is a mixture of amphipathic compounds capable of really acting as an "edge activator," thus destabilizing the lipid bilayer and increasing its deformability. On the other hand, Trc in low concentrations, as those used in this study, is mostly dispersed in the aqueous phase, thus being able to poorly interact with lipid bilayers. Oppositely, cineole, thanks to its lipophilicity and structure, can strongly perturb the liposomal membrane. Hence, the obtained results highlighted the influence of the different PEs on the physicochemical properties of the PEVs as a consequence of the diverse interaction with the PC bilayers.

Ex vivo diffusion experiments showed no permeation of minoxidil through the whole skin using both control and PEVs, but all PEVs were able to improve skin deposition in comparison to conventional liposomes (control). As shown in Fig. 6.3 the highest drug accumulation was found in the stratum corneum (SC), where PEV-1 (vesicles with Lab) and PEV-3 (vesicles with cineole) were about three times more efficient than control ($p<0.01$). On the contrary, PEV-2 (vesicles with Trc) slightly improved drug deposition in comparison to the control liposomes (by a factor of 1.3; $p<0.05$).

Thus, results of drug deposition were strongly correlated to the vesicle deformability and, hence, to the physicochemical properties of the PE.

To investigate in detail the role of the used PE, we compared minoxidil (trans)dermal penetration from PEVs to that from drug solutions with the "free" PE, used in the same concentration as in the vesicles (i.e., 2 %), and from a commercial minoxidil solution (control). When drug hydroalcoholic PE solutions were used, permeation of minoxidil through the newborn pig skin layers always occurred especially with Lab and Trc. On the contrary, PE solutions led to a decreased drug accumulation in the SC, and only cineole solution was able to increase drug deposition in the dermis. Moreover, the total amount of the drug delivered to the skin layers by the PE solutions was lower than that found with PEVs. This also indicated that the mechanism by which PEVs interact with the skin could not be a result of only the penetration-enhancing effect. Other phenomena might also have an influence on the penetration-enhancing effect of PEVs.

Therefore, the influence of skin pretreatment with empty PEVs and control was also studied. Results shown in Fig. 6.3 revealed that the pretreatment with PEVs reduced drug accumulation in the SC, but improved deposition in the dermis, leading also to an important transdermal delivery, which improved also in the control. Although these results should be seen as a proof of the enhancing properties of the deformable vesicles (Kirjavainen et al. 1996), the total amount of drug delivered to and through the skin by PE solutions after pretreatment with empty vesicles was lower than that found with the most deformable PEV-1 (Lab) and PEV-3 (cineole). On the contrary, the control liposomes and the Trc-containing PEV-2 showed a total skin delivery of the drug lower than obtained with the pretreatment. In conclusion, skin deposition improved in the following

Table 6.2 Determination of deformation index (DI) of vesicles containing different PEs (Labrasol® (Lab), Transcutol® (Trc), cineole). Each data is the mean ± SD; $n \geq 6$

Formulation	*z*-diameter (nm) before extrusion	*z*-diameter (nm) after extrusion	DI
Control	144 ± 3	127 ± 8	69
PEV-1 (labrasol)	202 ± 3	207 ± 16	400
PEV-2 (transcutol)	171 ± 6	177 ± 6	152
PEV-3 (cineole)	144 ± 3	140 ± 10	331

From Mura et al. (2009), IJP

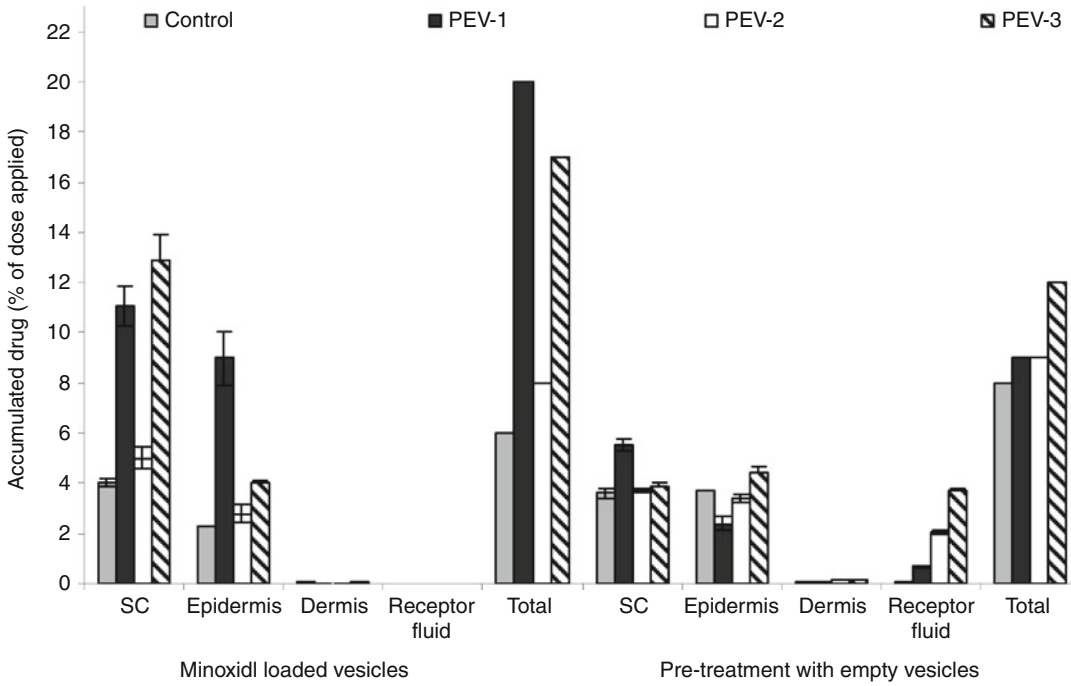

Fig. 6.3 Comparison of cutaneous and transdermal delivery of minoxidil after 8 h non-occlusive treatment with drug-loaded PEVs and control and pretreatment with corresponding empty vesicles (modified from Mura et al. 2009)

order: control < PEV-2 < PEV-3 < PEV-1. Overall results led us to suppose:

- PEVs do not simply act as penetration enhancers.
- PEVs can penetrate as intact vesicles the skin.
- PEVs form a depot in the SC from which the drug is slowly released.
- The composition of PEVs and therefore the nature of PE, affecting vesicle deformability, influence PEVs' behavior (Mura et al. 2009).

Fourier transform infrared spectroscopy (FTIR), attenuated total reflectance FTIR, and FTIR imaging verified that PEVs improved minoxidil delivery to the skin layers. Moreover, FTIR imaging confirmed the vesicle distribution in the SC (Mura et al. 2012).

Results showing that Trc-containing vesicles were not as efficient in minoxidil cutaneous delivery as Lab- and cineole-PEVs led us to suppose that Trc amount (2 %) could have been too low to obtain deformable and efficient PEVs. To ascertain this hypothesis, the influence of increasing Trc concentrations (5, 10, 20, and 30 %) on vesicle deformability and minoxidil cutaneous delivery was tested (Mura et al. 2011). To obtain stable minoxidil-loaded vesicles with the increasing amounts of Trc, a high concentration of soy PC (180 mg/ml) was needed. Comparing both empty and minoxidil-loaded vesicles, it was evident that Trc alone was not able to give any substantial improvement of vesicle deformability. However, the deformability of 5 and 10 % Trc-PEVs increased when minoxidil was also present in the formulation. Therefore, it was concluded that Trc can act as an edge activator only in the presence of a lipophilic molecule (such as minoxidil) that is incorporated in the bilayer, but is also soluble in Trc. Minoxidil dissolved in the Trc improved interaction of the PE with the vesicular bilayer. As Trc amount increased (>20 %), minoxidil solubility in the aqueous phase increased too with a consequent reduction of the amount of drug incorporated into the vesicular bilayer, which resulted in reduced vesicles' deformability. Viscoelastic behavior studies confirmed that the influence of Trc on the bilayer

properties is dependent on its used concentration and the solubility of the lipophilic drug. Ex vivo permeation studies performed in new born pig skin showed that conventional liposomes and 5 % Trc-PEVs gave the same drug distribution in the skin layers ($p<0.05$) with the drug mainly distributed in the SC (11 %) and epidermis (3 %). When the PE concentration was increased, a reduction of drug accumulation in the SC was observed with a concomitant increased deposition in the epidermis and dermis. Twenty percent Trc-PEVs (which have shown the lowest deformability) provided the highest drug retention in these strata, but the total drug amount delivered to the skin was less than that obtained with the control. Therefore, we concluded that association of Trc and PC does not give any synergic action capable of significantly enhancing minoxidil delivery to the skin (Mura et al. 2011).

The role of Lab in the enhancement of cutaneous availability of minoxidil loaded in PEVs was further studied to obtain information on its activity as a function of the preparation method. In fact, thanks to its amphipathic properties, during PEV preparation, it was added at different concentrations either in the lipid phase (0.5, 1, 2 % v/v) or in the water phase (2, 5, 10 % v/v). P50 was used as the phospholipid to form vesicles' bilayers incorporating minoxidil (2 % w/v). Non-dialyzed (ND) and dialyzed (D) vesicles were evaluated for ex vivo (trans)dermal delivery of minoxidil. Results showed improved skin minoxidil accumulation, influenced by both Lab concentration and preparation method (Caddeo et al. 2012). Indeed, 2 % Lab-PEVs prepared by adding the PE in the lipid phase were much more efficient than the corresponding formulation prepared by adding Lab to the aqueous phase.

ND and D Lab 2 %-PEVs (Lab in the lipophilic phase) provided the highest percentage (around 7 % each) of drug accumulation in the skin. An evidence that PEVs may enter intact the SC carrying their content and creating an improved pathway for free drug molecules throughout the skin was the finding that D vesicles had led to a similar or even higher percentage of drug deposition than corresponding ND ones.

6.2.3.2 Diclofenac

PEVs obtained with different concentrations of Trc were prepared and tested as carriers for diclofenac, one of the most used nonsteroidal anti-inflammatory drugs. Diclofenac was loaded into the vesicles either in the acid (DCF) or in the sodium salt form (DCF_{Na}), i.e., in the lipophilic or hydrophilic form, respectively (Manconi et al. 2011a).

PEVs and conventional liposomes were prepared using a mixture of enriched soy PC (P90G, 90mg/ml), DCF or DCFNa (1 %, w/v), and Trc (0, 10, 20, or 30 % v/v) in PBS (pH 7.0) used up to 100 % w/v. Oleic acid in small amount (1 % w/v) was also added to the lipid components as a charge inducer.

Deformability results, listed in Table 6.3, indicated that control liposomes and DCF_{Na}-loaded PEVs had roughly the same elasticity independently from Trc concentration used, whereas DCF-loaded PEVs had increasing elasticity as Trc concentration increased. In particular, DCF-loaded PEVs with 30 % Trc appeared approximately up to threefold more elastic than control liposomes and DCF_{Na}-loaded PEVs.

These results were in accordance with those obtained with minoxidil-loaded PEVs, i.e., only the simultaneous presence of the lipophilic DCF and Trc, here at high concentrations (20–30 %), led to increased vesicle elasticity, thus confirming that Trc-containing PEVs without a lipophilic drug are not highly deformable.

Nevertheless, the influence of Trc on the vesicle bilayer transition temperature (Tc) from gel to liquid-crystalline thermodynamic state was assessed by a rheological study using hydrogenated PC (Phospholipon 90H (P90H),

Table 6.3 Deformation index (DI) of empty, diclofenac acid, and sodium salt (DCF and DCF_{Na}) control liposomes (0 % Transcutol® (Trc)) and 10, 20, and 30 % Trc-PEVs

DI			
Trc %	Empty	DCF	DCF_{Na}
0	6.21 ± 1.24	5.33 ± 2.21	4.17 ± 1.73
10	5.71 ± 1.90	5.65 ± 1.72	5.79 ± 0.90
20	6.52 ± 3.04	11.87 ± 3.84	5.04 ± 1.38
30	10.16 ± 3.05	16.42 ± 2.41	5.16 ± 0.23

From Manconi et al. (2011a)
Each value represents the mean ± SD; $n=3$

Lipoid GmbH, Germany) because of the very low Tc (−15/−7 °C) of the non-hydrogenated P90G. Temperature-controlled rheometry in the oscillation mode has been demonstrated to be a valuable alternative to differential scanning calorimetry, and it was used to correlate the thermotropic behavior of semisolid liposomal preparations (Bender et al. 2002). Therefore, Tc was measured as a function of the complex viscosity variation: as temperature increased, the viscoelastic character of the gel-state bilayers decreased because of its enhanced fluidity (Manconi et al. 2011a, b).

Indeed, decreased Tc and peak area values were found as a function of the Trc amount in formulations (Table 6.4), as a consequence of a dose-dependent perturbation of the phospholipid packing by the cosolvent that fluidizes the vesicle bilayer. As its concentration increases, Trc may be partially incorporated into the bilayers interacting with only the methyl ends of the PC acyl chains, because of the steric hindrance of the polar head group of the Trc molecule and its short lipophilic chain (Adachi et al. 1995). Therefore, these outcomes indicate that Trc-PEVs were in a more fluid state in comparison with the conventional liposomes. Superior empty Trc-PEVs' fluidity (Tc decreased from 51.31 to 46.62 °C), undoubtedly due to the Trc presence, might facilitate the interaction between vesicles and the skin and even favor penetration of vesicles through the skin lipid bilayers, disorganized by the presence of "free" Trc molecules. To prove this hypothesis, ex vivo (trans)dermal experiments were carried out using DCF- and DCF_{Na}-loaded PEVs and also a commercial DCF_{Na} gel as control.

Table 6.4 Influence of Transcutol® (Trc) on vesicular lipid phase transition temperature (T_c).

Trc %	T_c (°C)	Temperature range (°C)	Peak area (au)
0	51.31 ± 0.056	11.24 ± 0.411	622 ± 36
10	49.78 ± 0.321	9.43 ± 0.416	302 ± 24
20	48.55 ± 0.926	7.78 ± 0.551	152 ± 15
30	46.62 ± 0.275	7.83 ± 0.568	76 ± 12

From Manconi et al. (2011a)
The width (temperature range) and the area under the peak of the complex viscosity spectra of the formulations are represented. Values represent the means ± SD; $n = 3$

As shown in Fig. 6.4, DCF and DCF_{Na} delivery was enhanced by PEVs in comparison with both controls, especially the commercial DCF_{Na} gel. Moreover, at the same Trc concentration, PEVs led to a DCF accumulation into the skin greater than or equal to that of DCF_{Na}, except for 30 % Trc-PEVs that provided a larger amount of DCF_{Na} in the SC and epidermis.

For DCF, the highest drug accumulation was always found in the epidermis, where 10 and 20 % Trc-PEVs enhanced drug deposition threefold compared to control liposomes. DCF and DCF_{Na} permeation also increased, but the transdermal delivery was always higher for the hydrophilic form of the drug. Moreover, results showed that drug accumulation and permeation were closely related to Trc concentration, thus showing that PEVs were more efficient than both controls in the dermal and transdermal delivery of DCF and DCF_{Na} and thus useful for locoregional treatment of inflammatory diseases.

In particular, comparison of the results from DCF and DCF_{Na}, showing the high deposition of the lipophilic form in the epidermis besides its increased transdermal delivery with increasing Trc concentration, led us to suppose that PEVs, or some of them at least, could be able to penetrate intact the skin forming a depot from which the drug can be released and then, free from the carrier, may penetrate deeper according to its physicochemical properties. Pretreatment studies were also conducted and reduced drug deposition and transdermal drug delivery were obtained. Overall results seem to confirm the dual effect of Trc that improves vesicular bilayer fluidity and also acts as a PE by reducing the barrier function of SC transiently, thus creating an easier pathway for the penetration of the highly fluidized vesicles.

A further proof of a potential synergistic mechanism of Trc and PEVs on the intercellular pig skin lipids was obtained by a qualitative study using CLSM during which penetration extent and localization of a lipophilic (β-carotene, β-C) and

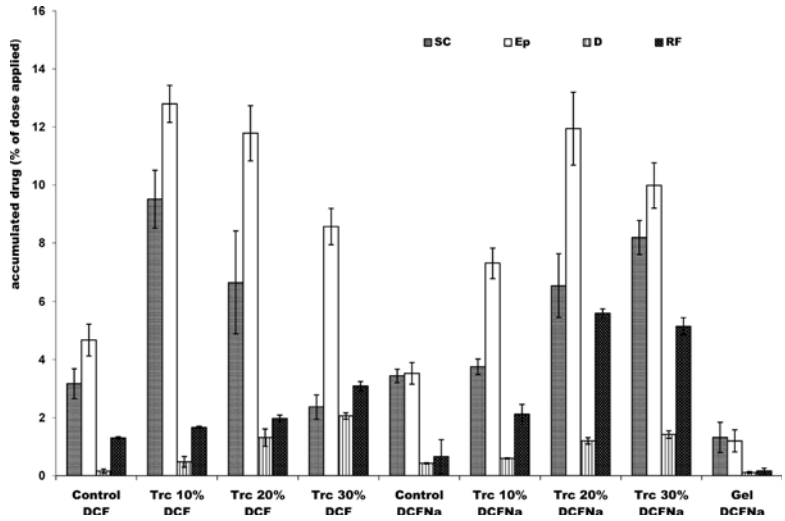

Fig. 6.4 Cumulative amount of diclofenac (DCF) and diclofenac sodium salt (DCF_{Na}), retained in and permeated through pig skin layers after 8 h non-occlusive treatment with PEVs and controls (conventional liposomes and commercial DCF_{Na} gel). *SC* stratum corneum, *Ep* epidermis, *D* dermis, *RF* receptor fluid. Each value is the mean ± SD; $n = 6$ (Modified from Manconi et al. 2011a)

a hydrophilic marker (5-(6)-carboxyfluorescein, CF), both loaded in 20 % Trc-PEVs and conventional liposomes, were visualized.

Figure 6.5 shows the different distribution of the two labels in the skin layers obtained with the two vesicle samples. In particular, images of the epidermis revealed the highest difference, where micrographs show a superposition of the green (CF) and red (β-C) fluorescence. This is particularly intense for PEV-treated skin, where large regions show a yellow fluorescence that clearly indicates that the hydrophilic CF and the lipophilic β-C reached the epidermis at the same time. Conventional liposomes led also to a superposition of the two markers, but the faded red fluorescence widespread overall this layer was indicative of a prevalent deposition of the lipophilic marker at this level, while the CF was predominant in the dermis layer, probably due to the faster permeation of this hydrophilic probe toward the deeper skin strata. The different distribution of the two probes with control and PEVs indicates a diverse mode of action of the vesicles. With control liposomes, the two markers' distribution was mainly regulated by their physicochemical properties, while using PEVs the markers' spreading was carrier mediated: hydrophilic and lipophilic markers reached mostly simultaneously the same place. Therefore, these results show the PEVs' superior ability to reach as intact vesicles the deeper skin layers.

Further, the influence of the vesicles' lipid composition on the dermal delivery of DCF_{Na} from Trc- PEVs was studied. For this purpose the vesicles were prepared by using the cheap commercial mixture of phospholipids P50, rich also in free fatty acids, while the Trc amounts were the same as in the previous study (i.e., 10, 20, and 30 %). As a consequence, the PEVs obtained possessed a highly negative surface charge and there was no need of using a charge inducer (i.e., OA). X-ray diffraction studies showed that vesicle structure is affected by the Trc amount and the drug loaded. Indeed, vesicles with 10 and 20 % Trc were unilamellar, while vesicles with the highest concentration of Trc were MLVs, independently from the preparation method (with or without sonication) (Manconi et al. 2012).

Only sonicated vesicles (smaller in size, Table 6.1) were tested for ex vivo transdermal penetration experiments, and the results showed that changing the lipid phase, a lower content of Trc (10 %) than that used with P90 in association with oleic acid, was enough to reach even better drug delivery than 20 % P90 PEVs. Also in this study, CLSM showed a superposition of the hydrophilic and lipophilic probes (5-CF and β-C) when delivered by PEVs in contrast to the co-labeled conventional liposomes which led to a prevalent deposition of 5-CF in the deeper skin layers, while β-C was especially accumulated in the upper skin region. Therefore, once more, it

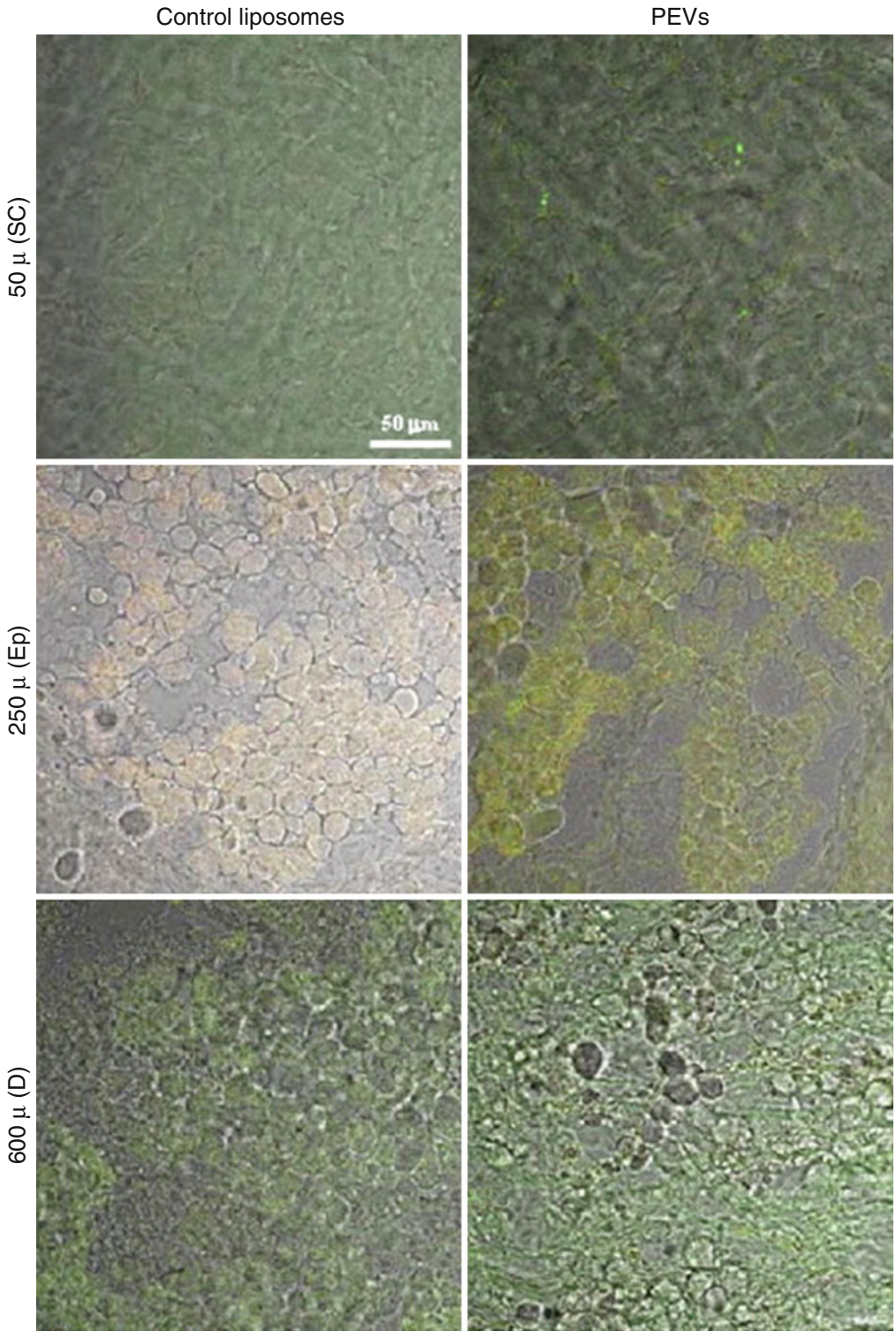

Fig. 6.5 CLSM images of sections parallel to the surface of the pig skin incubated for 8 h with fluorescent co-labeled conventional liposomes (control) and 20 % Transcutol® (Trc)-PEVs. Images show the distribution of green and red fluorescence, corresponding, respectively, to 5(6)-carboxyfluorescein and β-carotene, into the stratum corneum (*SC*), epidermis (*Ep*), and dermis (*D*). The bar is valid for all pictures (From Manconi et al. 2011a)

seems that Trc-PEVs could indeed act as drug carriers: at least some of the vesicles could cross intact the skin barrier by a synergistic mechanism between the vesicle carriers and the PE, which could have interacted with the SC intercellular matrix, thus creating an easier pathway for the penetration of the fluidized vesicles (Fig. 6.6).

Glycerosomes

Another type of new phospholipid vesicles containing an aqueous cosolvent is glycerosomes, which are obtained from different phospholipids by using high concentrations of glycerol (10–30 % v/v), a harmless and fully accepted compound for topical administration. Glycerosomes are versatile vesicular carriers that can be obtained by any of the different techniques commonly used for the preparation of conventional liposomes (Zaru et al. 2014, Manca et al. 2013).

They are fluid, soft vesicles with a spherical shape, as shown in Fig. 6.7, where the multilamellar morphology of the glycerosomes prepared by the thin film method is shown. They have been tested as carriers for different drugs, such as acyclovir and D-alpha-tocopheryl polyethylene glycol-1000 succinate (Zaru et al. 2014). They have also been tested for skin delivery of DCF_{Na} showing an improved cutaneous deposition of the drug as well as an enhanced drug flux through the skin in comparison with conventional liposomes (From Manca et al. 2013).

6.2.3.3 Tretinoin

Trans retinoic acid (TRA) was used as a model drug since the benefits of its incorporation into liposomes and niosomes on its dermal delivery have been already extensively studied (Sinico et al. 2005; Manconi et al. 2006). PEVs were prepared using the phospholipid mixture P50 with different hydrophilic PEs: Trc, Lab, PG, and decylpolyglucoside (Or), a commercial mixture of alkyl polyglucosides (Manconi et al. 2011b). In this study, *ex vivo* TRA skin deposition and permeation experiments were carried out by testing formulations (PEVs and conventional liposomes) having the drug both inside and outside the vesicles (ND), having TRA only inside (D), and using suspensions prepared by simply mixing TRA, phospholipids, and each PE. For all the tested formulations, the amount of TRA perme-

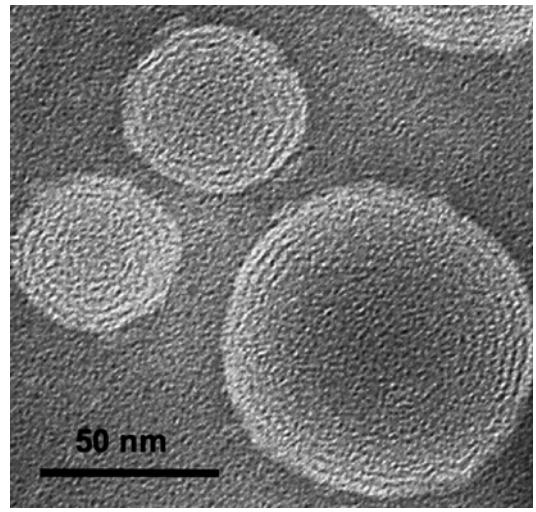

Fig. 6.7 Transmission electron microscopy image of glycerosomes (from Manca et al. 2013)

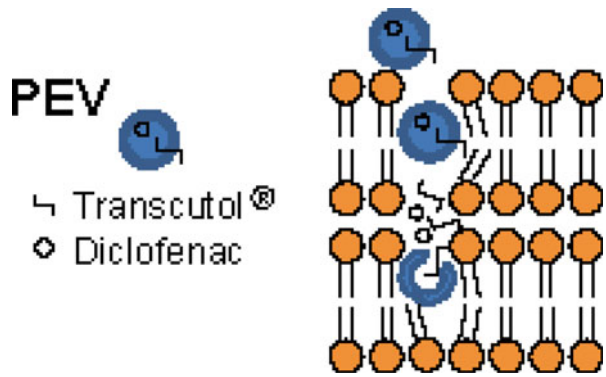

Fig. 6.6 Proposed mechanism of PEVs' penetration through intercellular skin lipids (From Manconi et al. 2012)

ated through the skin was lower than that accumulated in the skin, and it was higher for D formulations than for the corresponding ND vesicles. In addition, cutaneous TRA accumulation provided by D and ND conventional liposomes was very similar: low in the SC and epidermis, almost nil in the dermis. On the contrary, the TRA deposition provided by PEVs in all skin strata was always higher for D vesicles than for ND vesicles. Moreover, as shown in Table 6.5, the total amount of TRA delivered to the skin was about threefold higher with D PEVs than with D conventional liposomes, except for D PG-PEVs (only twofold higher). The TRA cutaneous accumulation from D formulations increased in the order: conventional liposomes < PG-PEVs < Trc-PEVs ≤ Or-PEVs < Lab-PEVs.

Moreover, when the suspensions were tested, the total amount of drug accumulated in the skin strata was always lower than that obtained with the corresponding vesicles, and the drug was especially deposited into the SC. Moreover, SEM studies showed the Or and Trc PEVs' ability to strongly interact with skin intercellular lipids causing an enlargement of this region (Fig. 6.8).

6.2.3.4 Quercetin

Different hydrophilic PE-containing PEVs were tested as carriers for quercetin, a bioflavonoid showing several pharmacological effects, including potent antioxidant activity (Chessa et al. 2011). Trc, PG, Lab, and PEG 400 were selected, and PEVs were prepared using P50 (60 mg/ml), quercetin (2 mg/ml), and a higher amount of the PE mixture in water (40 % v/v) than used before. Only using 40 % PE stable drug-loaded vesicles could be obtained because of the very low water solubility of quercetin, which could not be loaded into conventional liposomes.

Also in this case, drug delivery to the skin was studied in comparison with coarse drug dispersion of the same composition of the PEVs, and results confirmed that quercetin can be highly deposited in the skin (especially epidermis) only when the drug is loaded in the vesicles. In this study, the efficiency of PEVs for quercetin skin delivery was in the following rank: Lab-PEV < Trc-PEV < PG-PEV = PEG-PEV. Vesicles were also labeled with rhodamine-phosphoethanolamine to follow the fate of topically applied fluorescent bilayer marker by CLSM. Fluorescence was always higher in the

Table 6.5 Results of in vitro permeation study obtained using *trans* retinoic acid (TRA)-loaded PEVs and control liposomes, before (ND) and after dialysis (D) and TRA in a coarse dispersion of Phospholipon 50 (P50) and PE (MIX). Amount of TRA accumulated into the whole skin and permeated through the skin at the end of the experiment (8 hours); Local Accumulation Efficiency (LAC) values and transdermal flux (J)

Composition	TRA accumulated (μg/cm^2)	LAC	TRA permeated (μg/cm^2± SD)	J (μg/cm^2/h ±SD)
P50 ND	5.6	39	0.14 ± 0.01	17 ± 3
P50 D	4.1	30	0.14 ± 0.01	18 ± 3
P50 MIX	3.1	7	0.44 ±0.09	34 ± 4
P50/Or ND	2.9	16	0.17 ± 0.02	17 ± 2
P50/Or D	12.1	96	0.13 ± 0.02	14 ± 2
P50/Or MIX	3.1	10	0.33 ± 0.03	38 ± 4
P50/Lab ND	4.3	6	0.71 ± 0.01	88 ± 6
P50/Lab D	14.6	17	0.84 ± 0.10	101 ± 8
P50/Lab MIX	4.8	38	0.13 ± 0.01	15 ± 4
P50/Trc ND	7.8	23	0.33 ± 0.10	43 ± 5
P50/Trc D	13.4	14	0.76 ± 0.01	95 ± 9
P50/Trc MIX	4.6	60	0.07 ± 0.01	11 ± 2
P50/PG ND	6.8	4	1.64± 0.08	195 ± 14
P50/PG D	8.7	3	2.70 ± 0.20	315 ± 13
P50/PG MIX	2.3	18	0.12 ± 0.01	12 ± 3

From Manconi et al. (2011b)

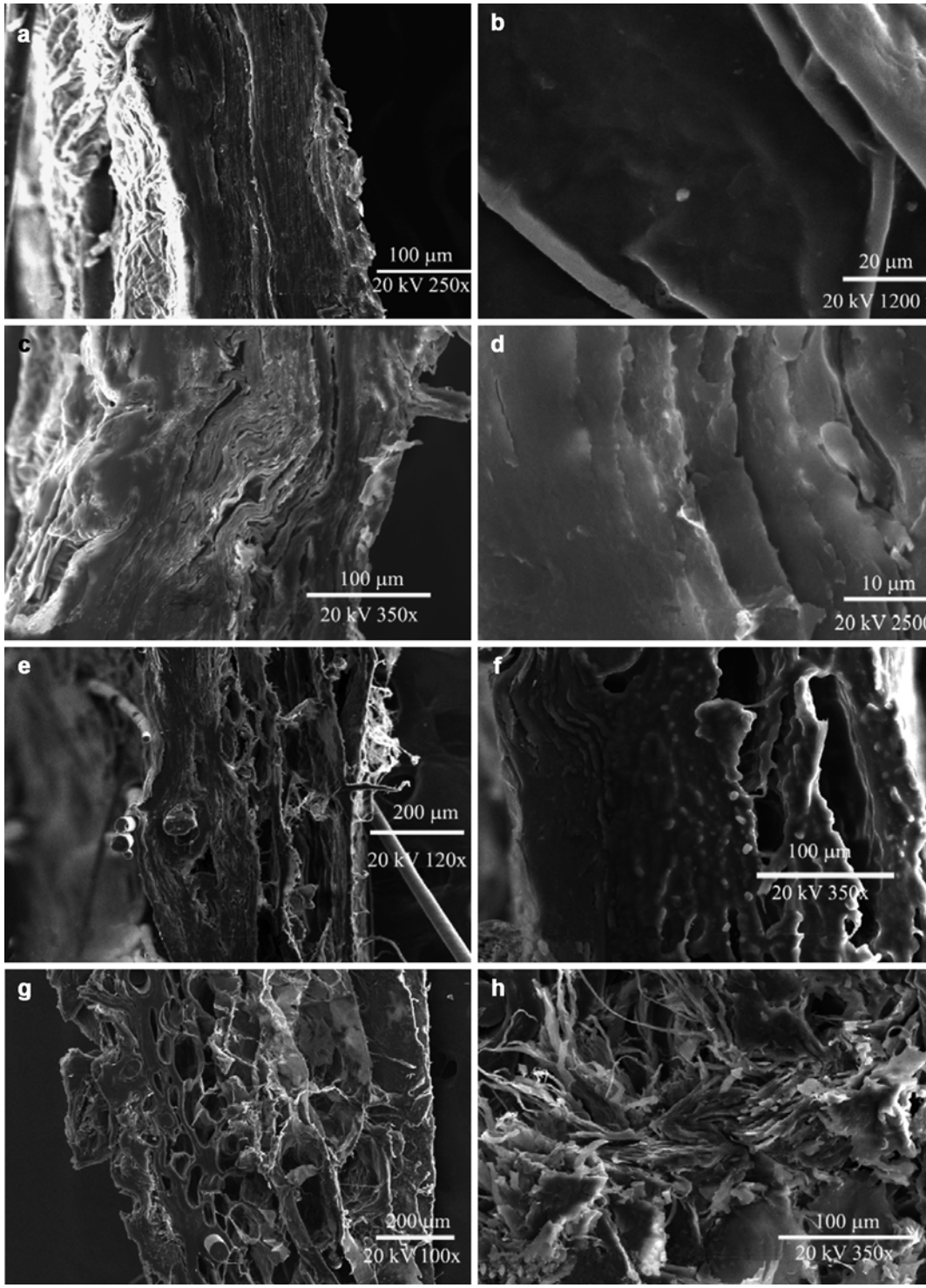

Fig. 6.8 SEM micrographs of the pig skin treated with phosphate-buffered dispersion of *trans* retinoic acid (TRA) (**a**, **b**), TRA-loaded liposomes (**c**, **d**), TRA-loaded decylpolyglucoside (Or)-PEVs (**e**, **f**), and TRA-loaded Transcutol® (Trc)-PEVs (**g**, **h**) after 8 h of application. Cross-sectional images taken perpendicular to the treated skin surface and morphology of skin surface are shown (*left and right column*, respectively) (From Manconi et al. 2011b)

SC than in the epidermis and dermis. PEG-PEVs showed a high fluorescence also in the epidermis, while PG- and Trc-PEVs promoted the marker accumulation in the dermis (Chessa et al. 2011).

Conclusion

Intensive research has led to the conclusion that PEVs are interesting carriers for dermal drug delivery. Their properties are strongly affected by the used PE and its capability to interact with the liposomal bilayer. Also the loaded drug plays an important role. Indeed, due to their structure, hydromiscible cosolvent PEs cannot insert deeply into the lipid bilayer, but interact mostly with the polar head group of the phospholipids. Therefore, these new vesicles are generally less deformable than those prepared with lipophilic and amphipathic PEs. However, they can enhance drug delivery into and through the skin by a synergistic mechanism among the PE, the carrier, and the intercellular skin lipids. In our opinion, this is one of the benefits of these vesicles.

Moreover, all PEVs are characterized by highly fluidized membranes that can penetrate into the skin barrier, thanks to the pathway created by the associated PE. Furthermore, vesicle preparation is simpler in the presence of PEs and leads to very stable nanocarriers.

Finally, all the used components of PEVs are biocompatible and safe. Therefore, these vesicles are promising, adaptable carriers for the safe and effective topical delivery of drugs.

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Stratum Corneum Lipid Liposomes: Drug Delivery Systems and Skin Models

7

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Contents

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7.1 Introduction

The use of liposomes as skin drug delivery systems has gained interest recently. They were shown to provide both localized and transdermal effects (El Maghraby et al. 2008). The effects of liposomes on the skin depended on the morphology and lipid composition. Vesicles made of various lipid compositions with different additives have been tested. Authors started with simple phospholipid liposomes with or without cholesterol forming vesicles which have been considered as the standard or traditional liposomes. The fabrication of vesicles was moved forward to incorporate surfactants into vesicular membrane to impart deformability and flexibility for the vesicles. Other groups started using vesicles tailored from stratum corneum lipids to produce the so-called stratum corneum lipid liposomes (SCLL). The later has gained interest in the area of skin drug delivery with the vesicles being employed as pharmaceutical carrier systems (Fresta and Puglisi 1996). In addition, great success is being reported for the SCLL when used as model membranes for the skin (El Maghraby et al. 2008; El Maghraby and Williams 2009). The proceeding sections will provide an overview of SCLL and their application as skin drug delivery systems and as model membranes.

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7.2 Skin Structure

The details of human skin structure and its barrier functions are well documented in literature (Scheuplein and Blank 1971; Elias 1981; Orland 1983; Barry 1983). The barrier function of the skin in the healthy and diseased state was also reviewed recently (Bouwstra and Ponec 2006). The stratum corneum (SC) is considered as the main barrier for drug permeation through the skin (Barry 1983). It comprises 15–20 layers of corneocytes embedded in the intercellular lipid matrix (Christophers and Kligman 1964; Christophers 1971). The SC is organized in the so-called bricks and mortar arrangement (Elias 1983), where the keratin-rich corneocytes (bricks) are embedded in the intercellular lipid-rich matrix (mortar). This arrangement is shown in Fig. 7.1 with the detailed structure of the intercellular lipid lamellae.

For any molecules applied to the skin, two main routes of skin permeation have been identified, i.e., the transepidermal and the transappendageal pathways (Scheuplein 1965). The later is believed to play minor role in transdermal drug delivery (Scheuplein 1967). The transepidermal pathway can be defined as the pathway where compounds permeate across the intact, unbroken stratum corneum. This pathway contains the intercellular route, which is a continuous but tortuous way through the intercellular lipids, and the transcellular route through the keratinocytes and across the intercellular lipids (Fig. 7.1, Barry 1991). As shown in the diagram (Fig. 7.1), the transcellular pathway requires not only partitioning through the keratin bricks but also across the intercellular lipids. Thus, the intercellular lipids are important for both pathways playing a major role in the barrier nature of the SC, even for hydrophilic compounds. This encouraged researchers to extract these lipids and study their role in depth. For example, skin permeability to water was significantly increased after removing skin lipids (Scheuplein and Blank 1971). Further, the variable permeability of the skin from different body sites can be related to total lipid content.

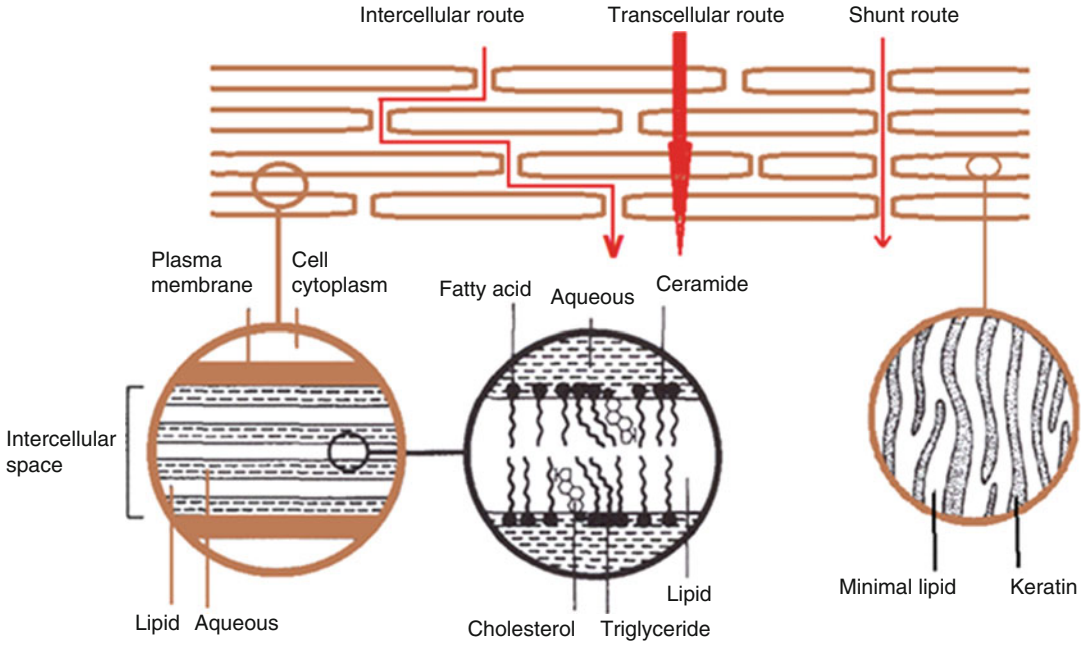

Fig. 7.1 Diagram of the brick and mortar model of the stratum corneum with a simplified lamellar organization of intercellular domains showing the major stratum corneum lipids. Also shown are the possible drug permeation pathways through intact stratum corneum: the transcellular, the tortuous intercellular, and shunt pathways (Modified from Elias 1981, Barry 1991)

Accordingly, the intercellular pathway is widely regarded as the main route of permeation of most compounds despite the relatively small surface area available for this penetration route (Albery and Hadgraft 1979; Guy and Hadgraft 1989). Being considered as the primary barrier to permeation of most drugs, extensive research is being conducted to understand the composition and organization of the structure of lipid domains of the SC.

During epidermal differentiation, the lipid composition changes from a polar character to a neutral mixture (Lampe et al. 1983). For whole stratum corneum, the major fractions are neutral lipids (78 %) and sphingolipids (18 %) together with a small amount of polar lipids. There is a considerable quantity (11 %) of nonpolar material present, such as squalene and n-alkanes. Both saturated and unsaturated fatty chains exist in all neutral lipid species, with unsaturated chains predominating except for the free fatty acids fraction. The ceramide (sphingolipid) fraction comprises primarily saturated fatty acid chains. The n-alkanes range in a bell-shaped distribution from C19 to C34 (Lampe et al. 1983). Thus, many lipid species exist in the horny layer, differing both in type and chain length; this complex lipid mixture forms bilayered structures (Fig. 7.1) (Barry 1987). These lipids have been isolated, and the lipid composition was approximated and proportionated before constructing the so-called SCLL.

7.3 Stratum Corneum Lipid Liposomes (SCLL)

The SCLL are morphologically similar to standard phospholipid liposomes; however, they differ in their composition. Their history has been started by Wertz et al. (1986) who prepared lipid vesicles from a lipid mixture approximating the lipid composition of the SC lipids. This mixture comprised 40 % ceramides (CE), 25 % cholesterol (CH), 25 % palmitic acid (PA), and 10 % cholesterol sulfate (CS). The prepared vesicles were as aforementioned termed SCL liposomes and were introduced into the field of skin drug delivery research as a drug delivery system and as a model membrane approximating the intercellular lipid lamellar structure which is believed to be the main barrier for drug absorption.

7.4 SCLL as Skin Drug Delivery Systems

The introduction of SCLL into the field of skin drug delivery was initiated with encouraging data. In the initial study, the authors employed the cutaneous herpes simplex virus guinea pig model to investigate the topical delivery of the peptide drug interferon from phospholipid liposomes, SCLL, and water-in-oil emulsion and aqueous solution. Both liposomes were more effective than water-in-oil emulsion and aqueous solution as revealed by reduced lesion scores after liposomal application. Application of interferon in SCLL produced greater remission of lesions than phospholipid liposomes. This finding highlighted the superiority of SCLL. The study was extended to investigate the effect of the method of liposome preparation. Vesicles produced by dehydration rehydration (DRV) method were more effective than those prepared by mechanical shaking or extrusion (Weiner et al 1989). The authors attributed the superiority of DRV vesicles to increased drug partitioning into the vesicle. The superiority of DRV was explained latter on the bases of increased entrapment efficiency by this method of preparation (El Maghraby et al. 2008). Shortly after that, the deposition of interferon into and permeation through skin was investigated after in vitro application of various liposome formulations. Tape-stripping technique was used and guinea pig skin was employed. Liposomes were shown to increase the deposition of the drug in the stratum corneum and deeper skin strata with no drug detected in the receptor. Again SCLL prepared by DRV were the superior with respect to improved skin deposition, compared to other formulations (Egbaria et al. 1990a). This finding suggested that the recorded pharmacological effect by Weiner et al. (1989) was due to improved skin deposition (i.e., a localizing effect) of the drug. The skin deposition of cyclosporine A from

various vesicular and non-vesicular formulations was similarly investigated using hairless mice skin (Egbaria et al 1990b). The deposition of cyclosporine A in the stratum corneum from various formulations was in the order of SCLL > phospholipid liposomes > O/W emulsion (emulsifier composition similar to lipid content of phospholipid vesicles) > hydroalcoholic drug solution (5 mg/ml in 40 % ethanol). The same study revealed reduced transdermal drug absorption from vesicles compared with the solution form (Egbaria et al 1990b). The superiority of SCLL was reflected also in the delivery of corticosteroids (Fresta and Puglisi 1997). The study compared the delivery of steroids from SCLL and phospholipid liposomes to that from an ointment. Both types of vesicles showed better skin deposition for various drugs compared with the ointment, with SCLL being better than other liposomal formulations. The anti-inflammatory effect of corticosteroids entrapped in vesicular structures correlated well with the skin accumulation results (Fresta and Puglisi 1997). This series of studies suggested that the SCLL exert their effect by improving the skin deposition of drugs and building a drug reservoir in the SC and deeper skin strata. This effect is known as the localizing effect of liposomes (El Maghraby et al. 2006, 2008).

Supporting those early findings, the potential of SCLL as skin drug delivery systems was probed after encapsulation of retinoic acid into these vesicles. The drug-loaded vesicles were incorporated into hydrogels before their in vitro application to rat abdominal skin. The liposomal formulation induced the formation of a drug reservoir in the skin which prolonged the effect of the drug in the viable skin (epidermis plus dermis). This was taken as an indication for enhanced local effect in the skin (Fresno Contreras et al. 2005).

Based on the reports relating the efficacy of liposomes as a transdermal drug delivery system with the fluidity and deformability of vesicles (El Maghraby et al. 1999, 2008), and on the hypothesis that high fusogenic activity of vesicles can improve the transdermal delivery of drugs from liposomes, the properties of SCLL were modulated (Tokudome et al. 2009). This modulation was conducted by changing the lipid composition of the SCLL with the goal of improving the transdermal drug delivery potential from such vesicles. The basic composition SCLL was taken as the starting point. The first attempt investigated the effect of the molar ratios of lipids of the SCLL. The effect of changing the alkyl chain length of CE was also assessed. In addition, the effect of fatty acid chain length and unsaturation was monitored. They measured the fluidity and fusogenic properties of vesicular membranes but did not conduct any permeation study. Based on the results of this investigation, the following useful points can be summarized:

- Increasing the proportion of CE increases the fluidity of liposomes, but this must be taken with caution as there is a maximum amount of CE above which liposomes will not be formed.
- The lipid composition producing vesicles with the highest membrane fluidity and maximum fusogenic activity is 45 % CE, 5 % CH, 5 % PA, and 45 % CS.
- The presence of a high concentration of CS contributes to the fusogenic properties of vesicles.
- CE with an alkyl chain length of 8 carbons produces liposomes with optimum fluidity and fusogenicity.
- The chain length of fatty acids did not alter the fluidity of liposomes' membranes; however, unsaturated fatty acids like linolenic acid (LA) can produce more fluid vesicles compared to the corresponding saturated fatty acids of the same chain length.

Based on the abovementioned information, the optimum liposome composition was suggested to contain 45 % CE, 5 % CH, 5 % LA, and 45 % CS (Tokudome et al. 2009). However, this needs further confirmatory investigation employing skin permeation studies.

7.5 SCLL as Skin Models

The establishment of the detailed structural model of the SC and its intercellular lipid lamellae (Fig. 7.1) encouraged the researchers to

employ liposomes as a skin model. This was based on the fact that the intercellular lipids are arranged in a bilayer structure with liposomes being the most appropriate organization mimicking this arrangement in vivo. Initial investigations employed standard phospholipid liposomes as a skin model (Beastall et al. 1988; Rolland et al. 1991; Bonina et al. 1994; Hadgraft et al. 1996; Inoue et al. 2001; El Maghraby et al. 2004; Auner et al. 2005), but the fabrication of SCLL by Wertz et al. (1986) opened the way for their application as a skin model. This section will summarize the applications of SCLL as a skin model membrane to investigate the mechanism of action of various skin penetration enhancers.

7.5.1 Studies Monitoring the Permeability of Vesicular Membrane or Partitioning of Compounds into the Vesicles

SCLL have been used to investigate the mechanism of action of 1-alkyl-2-pyrrolidones as skin penetration enhancers (Yoneto et al. 1995, 1996). This was achieved by investigating the effect of the enhancer on the release of a fluorescent marker from the vesicles. The results revealed an increase in the release of the fluorescent marker from liposomes after the incorporation of the potential enhancer into liposomes. This was taken as an indication of increased fluidity of the vesicular membrane and suggested that the compound could exert its penetration-enhancing effect by disruption/or fluidization of the intercellular lipid lamellae. It is important to indicate that the results of these investigations correlated with data recorded using hairless mouse skin which revealed penetration-enhancing effect of the tested compound (Yoneto et al. 1995).

The rate of calcein release from SCCLL was monitored and was taken as a measure for the stability of the vesicular membrane of SCLL. Destabilization of vesicular membrane is evidenced by high release rate. This process was used to investigate the mechanism of action of phospholipid liposomes (Kirjavainen et al. 1996). They incubated phospholipid vesicles with calcein-loaded SCLL and monitored calcein release. Liposomes containing dioleoylphospatidylethanolamine (DOPE) or lyso-phosphatidylcholine (lyso-PC) increased calcein release from SCLL indicating destabilization of the SCL liposomal membrane. These results were taken as an indication of the penetration-enhancing effect of phospholipid liposomes, and they were in correlation with the results of a pretreatment study conducted on human skin which showed enhanced permeation of the fluorescent marker following pretreatment with liposomes containing DOPE or lyso-PC (Kirjavainen et al. 1996). A similar study was conducted later, and it was useful in predicting the mechanism of action of phospholipid liposomes (Kirjavainen et al. 1999a).

The effect of liposomal phospholipids on fluidity of SC lipid bilayers and drug partitioning into them was evaluated, employing the SCLL as a model for human SC (Kirjavainen et al. 1999b). The results revealed that drug partitioning into SC lipid bilayers depends on the type of liposomal phospholipids. Thus, incorporating egg-PC, soya-PC, or DOPE into SCLL increased the partitioning of drugs into these SCLL, but incorporation of distearyl-PC did not change this partitioning. This was used to predict the effect of phospholipids on the skin as one of the possible mechanism for enhanced drug delivery to the skin by lipid vesicles. On the basis of this study, it was suggested that only fluid state phospholipids can disrupt the rigid structure of the skin lipids, thus increasing drug partitioning into the lipid phase. Increased drug partitioning was thus considered as a possible explanation for the phospholipid-enhanced skin permeation of drugs. Once again, this finding was confirmed by data recorded after a partitioning study employing human stratum corneum in vitro, which revealed increased drug partitioning into the SC after dipping into various liposomes (El Maghraby et al. 1999). These studies indicated the suitability of SCLL as a skin model. Thus, SCLL prepared from the extracted human stratum corneum lipids have been used to investigate the mechanism of action of skin penetration enhancers (Ibrahim

and Li 2010). The authors measured the uptake of various enhancers such as Azone, padimate O, and oleic acid by the SCLL. The degree of enhancer uptake by the vesicles was taken as an indication that the enhancer exerts its effect by modulating the lipoidal pathway of the stratum corneum.

In an attempt to mimic the transcellular penetration pathway which the drug has to pass through the keratin-rich cells and the intercellular lipids, proteoliposomes were prepared (Lopez et al. 1996). These were prepared using a mixture of skin lipids and proteins, extracted from pig SC. These vesicles were tested as skin models for studying the interactions of surfactants with skin. The alteration in the release of entrapped fluorescent marker from these liposomes after addition of surfactant was taken as a measure for the interaction of surfactants with the lipid membranes. They compared proteoliposomes with standard PC vesicles. The results revealed similar changes in bilayer permeability in the presence of the same amounts of surfactants, but different release kinetics were evident due to variation in the components of vesicular membrane through which the drug is permeating (Lopez et al. 1996).

7.5.2 Differential Scanning Calorimetric (DSC) Studies

DSC has been employed to investigate the thermal phase transition of human SC and to investigate the mechanism of action of skin penetration enhancers (Goodman and Barry 1985, 1986). Later on, standard phospholipid liposomes were extensively used as models for the same purpose using DSC (Beastall et al. 1988; Rolland et al. 1991; Bonina et al. 1994; Hadgraft et al. 1996; Inoue et al. 2001; El Maghraby et al. 2004; Auner et al. 2005). However, considering the lipid composition and organization of the SC (Fig. 7.1), it is clear that the organization of the SC lipids, in which there are alternating ceramides, cholesterol, cholesterol esters, and fatty acids, is completely different from that of the phospholipid liposomes (PLL) in which the phospholipids are arranged successively. This raises question marks about the suitability of PLL as a skin model and highlights the need for testing an alternative model. Kim et al. (1993) were the first to employ SCLL to investigate the mechanism of action of skin penetration enhancers using DSC. However, the superiority of SCLL was revealed later by El Maghraby et al. (2005) who compared SCLL with PLL as skin models to investigate the mechanism of action of penetration enhancers using high sensitivity DSC (HSDSC). They investigated a variety of enhancers such as Azone, padimate O, octyl salicylate, and 2-(1-nonyl)-1,3-dioxolane (ND) which is commercially known as SEPA™. This section will comment on the data recorded for ND as it produced different data with different vesicles. Pure PLL of DPPC showed typical thermotropic phase behavior with a pretransition endotherm between 35 and 36 °C and the main endotherm between 41 and 42 °C (Fig. 7.2). The HSDSC traces of untreated SCLL (Fig. 7.2) revealed three main transitions. The first (T1) was at 43.8 °C and was attributed to movement of cholesterol side chain. The second transition (T2) was detected at 61.7 °C and was considered to result from melting of the cholesterol-free regions. The last one T3 was seen at 70.1 °C and was linked to the breakup of the association of the head groups together with melting of cholesterol-rich regions.

Incorporation of ND, a dioxolane derivative, into DPPC liposomes abolished the pretransition and increased the transition temperature (T_m) of the main endothermic peak (Fig. 7.2). The latter effect suggests a stabilizing rather than perturbing effect on the vesicular membrane. This action indicates a penetration retarding effect. Chemically speaking, the recorded effect is acceptable as ND is a dioxolane derivative which can form hydrogen bonds with each successive PL molecule. However, it is difficult to classify ND as a penetration retarder when other researchers have reported an enhancing effect, shown also by thermal and spectroscopic analysis of ND-treated human SC (Diani et al. 1995; Morganti et al. 1999). Incorporating ND into SCLL abolished T1 and broadened the endothermic peaks and reduced the T_m of T2 and T3. This effect indicates membrane disrup-

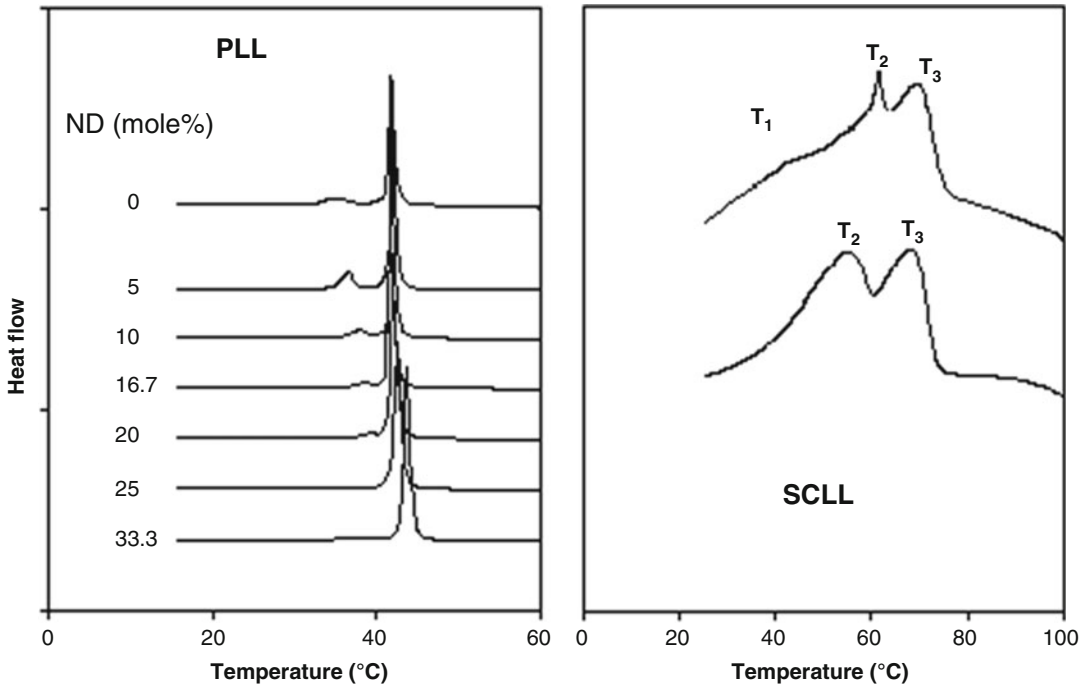

Fig. 7.2 Examples of the HSDSC traces of phospholipid liposomes (*PLL*), containing increasing concentrations of ND (*left diagram*) and traces of SCLL (*right diagram*) containing 0 (*top*) or 5 mmol of ND (*bottom*) (Modified from El Maghraby et al. 2005)

tion and a penetration-enhancing effect (El Maghraby et al. 2005), which agrees with the studies performed in the human skin (Morganti et al. 1999). This constructive study showed a defect in using PLL as a model of human skin and highlighted the importance of considering the lipid composition and the structural organization of the model membrane before making general conclusions.

Conclusion

SCLL have an increasing potential in dermal drug delivery. They can be used as a starting point to develop liposomes with a special drug delivery ability, but this requires extensive investigations to translate reported findings into skin drug delivery applications. In addition to the drug delivery function, the SCLL were shown to provide an excellent model for human SC. This model can provide rapid information about the mechanism of action of penetration enhancers and the potential effect of drug delivery carriers.

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Surface-Charged Vesicles for Penetration Enhancement

8

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8.1 Introduction

8.1.1 Skin Structure and Transport Mechanisms

The skin is the largest organ in the human body with a surface area of 1.8 m^2. However, it is a dynamic organ in constant regeneration. It has several functions and it also constitutes a physical barrier to the hostile environment. On the other hand, this barrier allows the passage of certain foreign substances into the body. This property has been used by numerous researchers, who focused their investigations on the possibility that drugs can pass through the skin, in order to obtain local or systemic effects (Betz et al. 2005; Honeywell-Nguyen and Bouwstra 2005; Rodríguez and Trujillo 2008).

It is well known that the human skin involves three structural layers, from the outer to the inner layers of the body: epidermis, dermis, and hypodermis or subcutaneous fatty layer. The epidermis is a stratified squamous epithelium that is constantly self-renewing. Keratinocytes are the cells of this epithelium with an average thickness of about 100 μm, which synthesize and express structural proteins and lipids during their maturation. Structurally, the epidermis is made up of five layers or strata: stratum basale, stratum spinosum, stratum granulosum, stratum lucidum, and *stratum corneum* (SC) as shown in Fig. 8.1.

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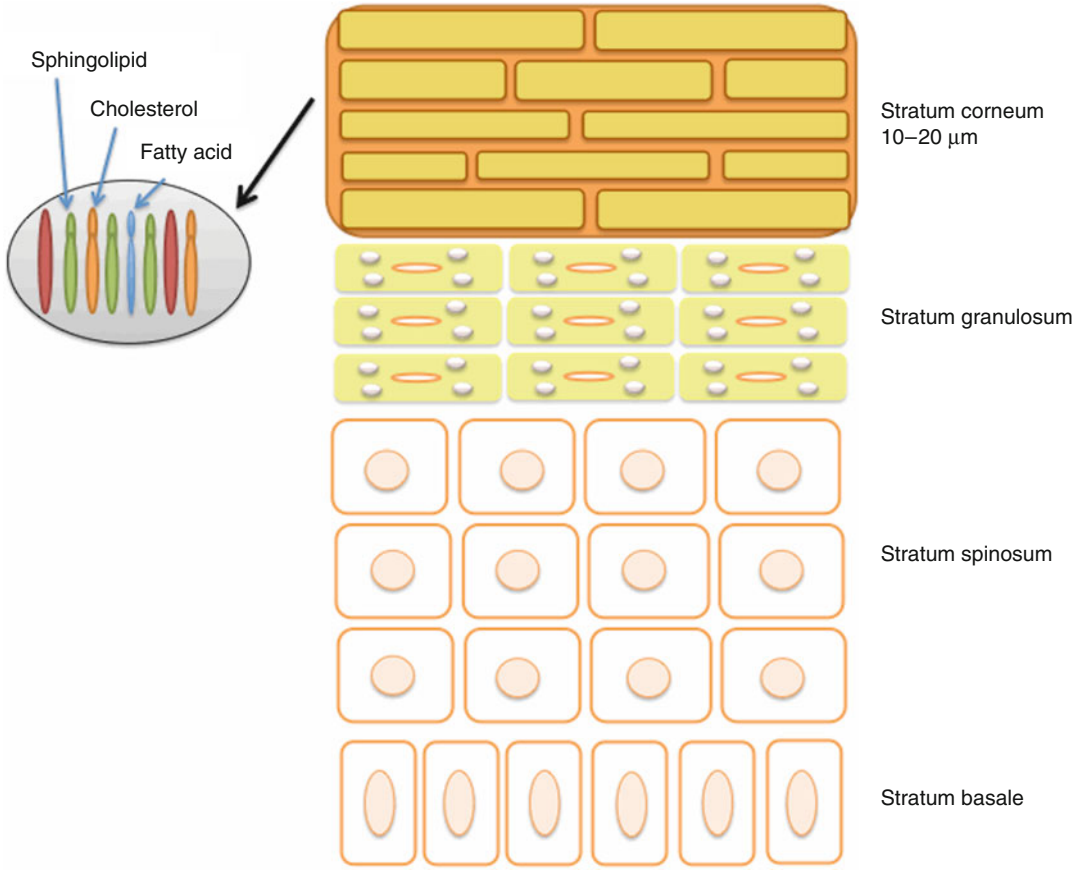


Fig. 8.1 Representative diagram of the skin structure and the main components of the stratum corneum

The main barrier for diffusion through the skin is the outermost layer of the skin, the SC, which consists of 15–25 layers of flattened, stacked, and hexagonal cells. This layer is composed of keratin-filled corneocytes within an intercorneocyte matrix. Corneocytes represent the last stage of the keratinization process and they are organized in a multilayered "brick and mortar" structure (essential to maintain the function of being an effective barrier to water loss), where cells (bricks) are embedded in a mortar of lipid matrix, composed of an intracellular lipid mixture of polyunsaturated fatty acids, cholesterol, and ceramides (Williams 2003). The removal of the superficial layers of the SC causes a remarkable increase in the permeability to water and other compounds, as reported in the literature (Elias et al. 1987; Martini 2005; Bouwstra and Ponec 2006).

The main obstacle to transdermal delivery is the efficient barrier properties of the SC. Their compact structure and the lipids play an important role in these barrier properties. There are three possible pathways by which a molecule can pass across the SC layer (Fig. 8.2):

(a) Trans-appendageal pathways: through the sweat glands and across the hair follicles with their sebaceous glands
(b) Intercellular pathways: via the lipid matrix between the corneocytes
(c) Transcellular pathways: across the corneocytes and the intercellular lipid matrix

The intercellular and transcellular pathways in transdermal drug delivery depend on the diffusivity of the molecule in lipids and proteins, the partitioning of the drug molecule between these

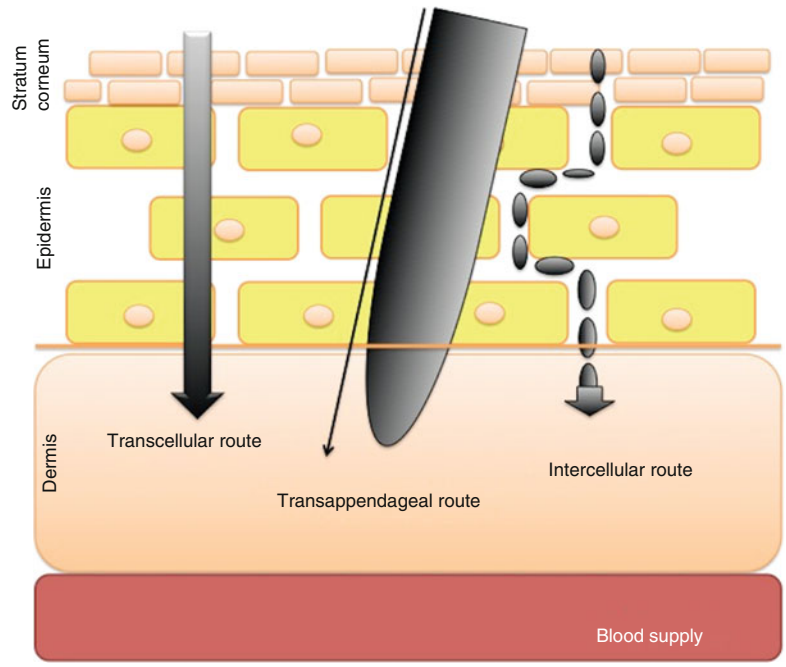


Fig. 8.2 Representative diagram of the main pathways for transdermal drug delivery

domains, and the geometry of the SC. Although most of the molecules undergo the passage through the membrane by the three mechanisms, the intercellular pathway is nevertheless considered the main route for permeation across the SC (Moser et al. 2001; Benson 2005).

8.1.2 Liposomes as Penetration Enhancers

Originally, liposomes were used as models of biological membranes. In 1965, Alec Bangham described that when phospholipids were dispersed in an aqueous medium, these structures were spontaneously formed.

Liposomes may be defined as spherical self-closed vesicles formed by phospholipid bilayers, which enclose part of the surrounding aqueous volume in their inner cavity. They are composed of concentric bilayers formed from self-assembly of amphiphilic phospholipids that allow the encapsulation of the drug inside the vesicle. Cholesterol can be incorporated in the formulation to increase the rigidity or stability of the membrane (Antimisiaris et al. 2008). These vesicles show a wide size distribution, ranging from 20 to 5000 nm in diameter depending on the composition and preparation method. Other subsequent procedures such as extrusion and sonication permit us to obtain narrower vesicle size distribution (Maestrelli et al. 2005, 2006).

Hydrophilic drugs can be entrapped inside the aqueous compartment. Furthermore, hydrophobic or charged hydrophilic drugs can be entrapped in the lipid bilayer due to hydrophobic and/or electrostatic interactions (Martin and Lloyd 1992). This property constitutes a clear advantage in the use of these systems to entrap substances of different physicochemical characteristics (Maestrelli et al. 2009; Villasmil-Sánchez et al. 2010).

Liposomes possess unique properties due to the amphiphilic nature of the lipids, which makes them suitable for drug delivery. Although liposomes have been extensively studied as promising carriers for therapeutically active compounds, some of the major drawbacks for liposomes parenterally administered are the rapid degradation due to the reticuloendothelial system (RES), primarily in the liver, and inability to achieve sustained drug delivery over a prolonged period of time. Moreover,

liposome-based systems are known to possess limitations such as instability, short half-life, and rapid clearance, being the rate of clearance dose dependent and varying according to the size and surface charge of the liposomes (González-Rodríguez and Rabasco 2011; Mufamadi et al. 2011). On the other hand, several disadvantages of using liposomes topically lie in their difficulty to penetrate intact across the skin and the stability of the formulation, because liposomes should be incorporated into a suitable vehicle to achieve the viscosity and consistency desirable for application (Patel Meghana et al. 2012).

It is generally accepted that classic liposomes are of minor value as transdermal drug delivery systems due to the fact that they do not penetrate deeply into the skin, but rather remain confined to the upper layers of the SC (Touitou et al. 2000; Betz et al. 2005). Several hypotheses have suggested that most conventional vesicles disintegrate at the skin surface and their molecularly dispersed components penetrate into the intercellular lipid matrix. Lipid components are then mixed with the SC lipids modifying the lipid lamellae and inducing new structures, permitting them to act as percutaneous drug penetration enhancers (Coderch et al. 1999).

In recent years, there has been much interest in delivering drugs to and through the skin using modified carrier vesicles. Different types of lipid vesicles are formulated depending on the additives used for preparation: Transfersome™ (Cevc et al. 1996; Cevc and Blume 2001), flexosomes (Maestrelli et al. 2009), ethosomes (Touitou et al. 1997; Paolino et al. 2005), niosomes (Uchegbu and Vyas 1998; Choi and Maibach 2005), vesosomes (Mishra et al. 2006a), invasomes (Dragicevic-Curic et al. 2008, 2009; Subongkot et al. 2012), and polymerosomes (Rastogi et al. 2009). Transfersome™, ethosomes, invasomes, and niosomes will be widely described in separate chapters of this book. However, since Transfersome™ and flexosomes were often used to investigate the influence of vesicle's surface charge on the drug delivery from vesicles, they will be described in brief in this chapter.

Deformable liposomes or Transfersome™ (Idea AG, Germany) are lipid vesicles formed by phospholipids and a surfactant named "edge activator," which destabilizes the lipid bilayer of the vesicle and increases its deformability (Cevc and Blume 2001). In 1992, Cevc and Blume developed this generation of elastic vesicles and suggested that the transport of Transfersome™ is driven by the osmotic gradient across the skin, i.e., the driving force for the movement of these liposomes is presumably generated by the hydration gradient across the skin, which varies from 15 to 20 % water content in the SC up to 70 % in the *stratum granulosum* (Cevc et al. 1996; Benson 2005; Honeywell-Nguyen and Bouwstra 2005; El Maghraby et al. 2008).

Flexosomes or flexible liposomes represent vesicles similar to Transfersome™. They are small-sized unilamellar vesicles (80–250 nm) containing a biocompatible membrane softener in the liposomal bilayer for targeted and noninvasive delivery of agents into and through the skin. Molecular agents such as DOTAP (1,2-dioleyl-3-trimethylammonium-propane), linoleic acid, and surfactants can be added to the formulation to increase the flexibility of vesicles (Maestrelli et al. 2009). There are neutral, anionic, and cationic flexosomes. However, anionic flexosomes are less stable than neutral and cationic.

Flexosomes overcome the skin barrier by opening intercellular hydrophilic pathways through the skin, i.e., the driving force for the movement of these liposomes is presumably generated by the hydration gradient across the skin as in the case of Transfersome™.

Low-molecular-weight heparin (LMWH)-loaded flexible liposomes were developed for transdermal delivery. In vitro studies demonstrated a 2.6-fold higher permeability coefficient with *flexosomes* compared to ethosomes, so their deformability was higher than that of ethosomes (Song et al. 2011).

8.2 Surface-Charged Liposomes as Penetration Enhancers

It is well known that the physicochemical characteristics of the liposomes, such as the fluidity, lamellarity, elasticity, and size, are important determinants

for their dermal drug delivery properties. In addition to these characteristics, several authors have outlined that drug penetration can be influenced by modifying the surface charge of these vesicles (Ogiso et al. 2001; Manosroi et al. 2002).

As was described in a previous section, the lipid layer in the SC contains a high ratio of negatively charged lipids and the skin may act as a negatively charged membrane (Sinico et al. 2005; Yoo et al. 2008). This is why many researchers have stressed that drug penetration can be influenced by modifying the surface charge of liposomes, because this parameter can affect the transcutaneous diffusion of drugs. Considering negatively charged vesicles, researchers have generally postulated that a higher flux than positively charged counterparts exists, which in turn can improve drug accumulation in the superficial skin strata (Manosroi et al. 2004). On the other hand, positively charged formulations cause an intensive adsorption of the negatively charged corneocytes of the SC, which enhances the retention time and the bioavailability (Piemi et al. 1999; Manosroi et al. 2004). However, results in the literature are contradictory.

The use of charged liposomes has been generally recommended, since the adsorption of liposomes onto the skin is due to physical (electrostatic, hydrophobic, etc.) forces.

In an initial approach, it is generally accepted that the positive charges on the surface of liposomal formulations could bind to the negative charges of the SC, the drug remaining as a depot in the outer layer of the skin. Moreover, researchers have reported that negatively charged vesicles mixed with the lipids of the SC were able to induce ultrastructural changes due to an impaired barrier function in the deeper layers of this stratum, providing an enhancer effect of the drug to the deeper skin layers. However, in some cases, the physicochemical properties of the drug, such as ionization constant, solubility, amphiphilic characteristics, etc., can influence the drug permeation behavior.

In the next paragraphs, some of the strategies reported in the literature to provide charges (positive or negative) to these lipid vesicles and their effect on drug permeation will be discussed.

8.2.1 Charged Lipids

Phospholipids are the main components of liposomes. Some of them are amphiphilic lipids consisting of a rather polar head group and comparably apolar residues. The important role of water in the membrane structure is explained by the fact that hydration of lipids leads to significant changes in thermodynamic parameters of their main phase transition depending on headgroup structure. The ordering of the water molecules at the membrane surface may be the reason for the main part of dipole potential at the membrane boundaries and the hydration forces. The addition of water-soluble solutes into liposome dispersions may alter the interaction between the phospholipid molecules at the bilayer surface. In addition, in a lipid bilayer, some of the constituting lipid molecules may carry a charge due to dissociation of their ionizable groups, and the charge amount could depend on pH. Therefore, the characterization of the surface charge properties under various conditions is of fundamental importance for liposome applications.

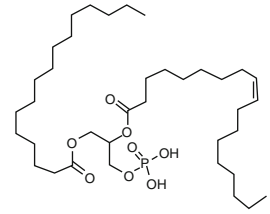
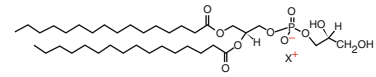
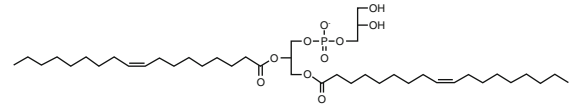
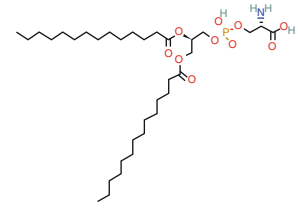
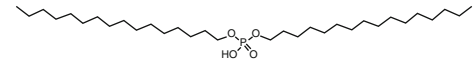
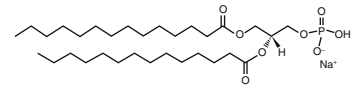
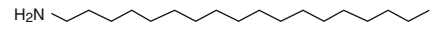
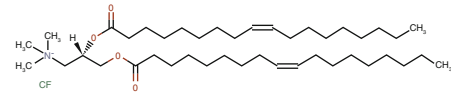
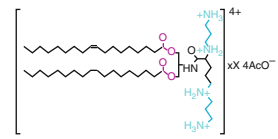
Table 8.1 summarizes some positively and negatively charged phospholipids used to enhance the skin permeation of hydrophilic and lipophilic drugs.

The effect of the charged-lipid presence in the bilayer on the skin permeation of drugs has been widely studied. However, the physicochemical properties of the encapsulated drug could modify the permeation behavior of these charged vesicles. Drug solubility and ionization ratio in the pH conditions of the aqueous compartment of vesicles can alter the surface charge and the electrostatic and hydrophobic interactions with the SC.

8.2.1.1 Hydrophobic Non-ionizable Drugs

When drugs non-ionizable at skin pH and lipophilic drugs are formulated in charged-lipid vesicles, a general behavior is observed in terms of permeation enhancement. Studies by Ogiso et al. (2001) revealed that the in vitro and in vivo percutaneous permeation of drugs such as betahistine, ethosuximide, or melatonin from negatively

Table 8.1 Chemical structures of some charged lipids and phospholipids used to enhance drug permeation

Negatively charged
POPG (Palmitoyloleoyl - phosphatidylglycerol) 
1,2-dihexadecanoyl-sn-glycero-3-[phospho-(1-glycerol)] 
DOPG 
DMPS 
Dicetyl phosphate (DCP) 
DMPA (1,2-dimyristoyl-*sn*-glycero-3-phosphate (sodium salt) 
Positively charged
Stearylamine 
DOTAP (N-[1- (2,3-Dioleoyloxy)propyl]-N,N,Ntrimethylammonium) 
DOSPER (1,3-Di-Oleoyloxy-2-(6-Carboxy-spermyl)-propylamid) 

charged liposomes (containing dicetylphosphate (DCP)) was higher than from positively charged liposomes (with stearylamine (SA)). Histological studies revealed that the negatively charged liposomes (whose charge was maintained because non-ionizable drugs at skin pH were loaded) diffused to the dermis and the lower portion of hair follicles through the SC. Thus, the rapid penetration of negatively charged liposomes would contribute to the increased permeation of drugs through the skin.

Other studies evaluated the effect of surface charge of liposomes on the permeation of amphotericin B showing that positive liposomes (with SA) provided the highest amount of drug in the SC, while the negative liposomes (with DCP) delivered the highest amount of amphotericin B in the viable epidermis (Manosroi et al. 2004). The low capacity of ionization of the drug at skin pH may contribute to the same behavior previously explained.

Gillet et al. (2011) recently published a paper in which the influence of surface charge of liposomes on the skin delivery of betamethasone and its dipropionate salt was studied. With the same justification as the previous examples, they explained that the presence of a negative charge in the lipid bilayer of vesicles (as DCP or dimyristoyl phosphatidic acid (DMPA)) is enough to enhance the penetration of the encapsulated drug.

Other studies have been carried out by simultaneously treating the skin with high-voltage electric pulses, which increase the number of pores created (Sen et al. 2002). It appears that negatively charged lipids (1,2-dioleoyl-3-phosphatidylglycerol (DOPG) and 1,2-dioleoyl-3-phosphatidylcholine (DOPC)) are necessary to enhance transport, because they are responsible for the increase in the lifetime of the electropores. The potential application of this technology in achieving transdermal delivery of small and large drug molecules (such as insulin) is considerable.

8.2.1.2 Hydrophobic Ionizable Drugs

For lipophilic drugs, but ionizable in the formulation medium, the entrapment into cationic vesicles can provide a permeation enhancer effect when formulation is topically applied. This was demonstrated by Montenegro et al. (1996) by using retinoic acid as a lipophilic but ionizable drug. Certainly, positively charged liposomes (formulated with SA) provided greater drug skin permeation compared to neutral or negatively charged liposomes containing 1,2-dipalmitoyl-sn-glycero-3-phosphoserine (DPPS) as an anionic phospholipid. The greater retinoic acid skin permeation observed using positively charged liposomes could be attributed to a greater accumulation of this type of liposomes within the SC, probably due to the negative charge of the skin surface. Once in the SC, liposomal bilayers can mix with the SC lipids forming a lipid depot in this skin layer. The negatively charged drug at skin pH (pKa 4.78) interacts with the positively charged phospholipid via electrostatic interaction, giving rise to an enhanced passage to the viable epidermal layers.

More recently, Kitagawa and Kasamaki (2006) developed cationic liposomes for transdermal delivery of this drug by using a double-chained cationic surfactant (DOTAP) and phosphatidylcholine (PC) to obtain these liposomes.

These results suggest the potential of the use of cationic liposomes for intradermal delivery of lipophilic drugs.

The same conclusion was reached by Sinico et al. (2005) in their studies on the influence of surface-charged vesicles on tretinoin permeation rate and retention of the drug in the outer layers of the SC or to accumulate in the inner layers. In this case, the negatively charged lipid was DCP. Results indicate that the drug release and skin permeation occurred rapidly, especially when the drug was delivered from positively charged liposomes. In addition, the tretinoin transport rate was higher in these vesicles than negatively charged liposomes. The enhancement in the drug diffusion through the skin could be explained as the consequence of ion pair formation between the organic acid tretinoin and SA counter ions, due to the partial ionization of tretinoin at pH 5.5 (pKa=7.85). As a consequence, SA acts by promoting drug delivery through the skin. It was possible to visualize a higher retention of tretinoin in the SC, in particular when negatively charged liposomes were used.

Liposomes have been widely used to enhance transfollicular delivery of low-/high-molecular-weight and hydrophilic/lipophilic compounds. Han et al. (2004) developed the transfollicular Adriamycin delivery system using cationic liposomes including DOTAP that improved the delivery amount and penetration of drug into the follicles and skin. In addition, to accelerate delivery, iontophoresis was combined with the cationic liposome, suggesting that the combinative system has a significant synergistic effect on transfollicular delivery of Adriamycin. As this drug itself is a cationic molecule, when iontophoresis was combined with cationic liposomes, it showed excellent transfollicular delivery. In addition, this process was enhanced by the increased positive charges of the cationic liposome, adding multication additives, such as DOSPER, spermine, and protamine.

More recently, Dragicevic-Curic et al. (2010) carried out the encapsulation of temoporfin (mTHPC) into charged flexosomes. This drug, because of its chemical structure, gives apparent pKa values of 3.45, 1.45, and 10, the last one corresponding to the phenolic groups (Bonnett et al. 2001). As reported by the aforementioned authors, cationic flexosomes provided the highest accumulation of mTHPC in the deeper skin layers. The highest total penetration-enhancing effect was also ascribed to cationic flexosomes, since they delivered the highest mTHPC amount to the whole skin.

Maestrelli et al. (2010) used the combined approach of cyclodextrin complexation in charged liposomes to increase the entrapment efficiency of lipophilic drugs, such as benzocaine and butambene. The double-loading technique incorporating the drug into the lipophilic phase and the aqueous compartment was employed. They demonstrated that the vesicles charged with SA enclosing highly ionized drugs at pH 5.5 (pKa 2.8 and 2.5 for benzocaine and butambene, respectively) have an enhanced permeation effect with respect to the non-charged liposomes (Maestrelli et al. 2010).

8.2.1.3 Hydrophilic Ionizable Drugs

Interesting results were reported by Song et al. (2006) when charged flexosomes containing a water-soluble and negatively ionized drug, a low-molecular-weight heparin (LMWH), were developed. This substance generally penetrates the skin very poorly due to its relatively large molecular weight, besides the negative charge and hydrophilicity. It was demonstrated that the higher entrapment of LMWH into cationic flexosomes (made with DOTAP), due to an electrostatic interaction, significantly increased drug delivery through the skin with respect to anionic vesicles (composed of DCP). Several mechanisms have been postulated to justify why cationic flexosome enhances LMWH accumulation in viable skin, but the following might be possible: when the drug-loaded vesicle (which has a lower cationic surface charge) penetrates into the skin under a transdermal hydration gradient and reaches the wet and deeper viable skin layer, it thus acts as a local reservoir filled with the LMWH vesicle. There, a fusion between the flexosome bilayer and the skin occurs by the lipase activity in viable skin. Because of the hydrophilic properties of the drug, a tendency to leave the lipid vesicle in the viable skin exists, also following a concentration gradient.

Villasmil-Sánchez et al. (2010) recently investigated the effect of the charge inducer agent on the zeta potential values between negatively and positively charged liposomes. In accordance with several authors, they concluded that DCP incorporated in liquid-crystalline PC bilayers is randomly distributed on the plane of the bilayer. Furthermore, the distribution of this negatively charged phospholipid between the two halves of the bilayer is uniform. However, this pattern was not reproduced with liposomes containing SA, where a possible asymmetrical distribution of SA in the bilayer was obtained. In addition, several authors have reported the capacity of SA to escape easily from the lipid bilayer and to protect its hydrocarbon chain from the hydrophilic environment: SA is organized in micelles, undergoing a rapid segregation into the medium, which change the surface charge density (Casals et al. 1998).

Figure 8.3 shows an illustrative representation of charged-lipid behavior on drug permeation and its effect on enhancement mechanism.

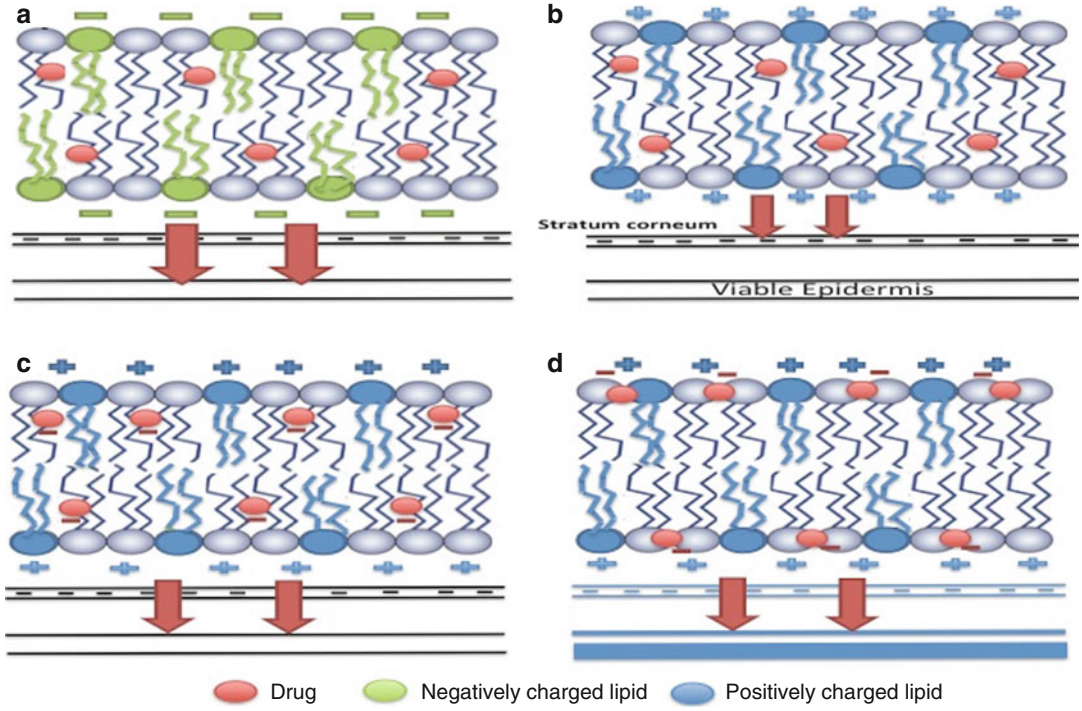


Fig. 8.3 Schematic plot to visualize the effect of charged-lipid behavior on drug permeation and its effect on enhancement mechanism. (**a**, **b**) Hydrophobic non-ionizable drugs; (**c**, **d**) Hydrophobic ionizable drugs

8.2.2 Charged Surfactants

It is evident that the physicochemical characteristics of the liposomes such as fluidity, lamellarity, elasticity, size, and charge are important determinants due to their dermal drug delivery properties.

Surfactants can increase the penetration of drugs into the skin, probably due to their ability to alter the barrier function of the SC. In addition, the presence of surfactants such as cholate, deoxycholate, sorbitan alkyl esters (Span®, Croda International PLC), polysorbates (Tween®, Uniqema), glycyrrhizinate, and bola-surfactant in the liposomal bilayer can disturb the packing of the liposomal bilayer, which might increase the deformability of the vesicles, independently of the phase transition temperature of the lipid bilayer (Paolino et al. 2007). In this section, some comments about the influence of the charged surfactants on drug permeation will be summarized.

The inclusion of membrane softeners, such as bile salts (sodium cholate, sodium deoxycholate, sodium taurocholate), into the PC membrane, allows the vesicles to adjust their shape to fit into pores smaller than their size and surface hydrophilicity which makes them able to cross the skin following the hydration gradient across the skin (Cevc and Gebauer 2003).

Oleic acid has been used by some research groups as a bilayer component of deformable liposomes for transepidermal drug delivery (Choi and Maibach 2005; Srisuk et al. 2012). The apparent pKa for this monounsaturated fatty acid in monoolein is within the physiological pH range of the intestine and depends somewhat on composition (Salentinig et al. 2010). The increased skin permeability by oleic acid is related to a selective perturbation of the intracellular lipid bilayers present in the SC. OA can dramatically change the morphology and the density of epidermal Langerhans cells and resulted in the generation of pores on the surface of epidermal

corneocytes. It is interesting to note that the permeation enhancement process is favored when lipophilic non-ionized drugs are entrapped in the vesicles, such as estradiol (Choi and Maibach 2005) and methotrexate (Srisuk et al. 2012).

Carrer et al. (2008) have described the presence of a transepidermal shunt through pig skin and evidence the accumulation of deformable liposomes in junctions between corneocyte clusters. They have speculated about the possibility that these structures may act as hydrophobic drug reservoirs. In addition, and regarding liposome composition, the presence of lysophosphatidylserine (LysoPS) as a negatively charged edge activator surfactant allows a more efficient penetration of drugs into the deeper layers of the skin.

The influence of charge and lipid concentration on the in vivo percutaneous absorption of a model compound, methyl nicotinate, from DDAB18 (didecyldimethylammonium bromide) liposomal vesicles was investigated by Puglia et al. (2005). Methyl nicotinate was chosen as model compound since it was partially dissociated at skin pH (pKa 3.2) and is capable of causing cutaneous erythema, the intensity and duration of which were proportional to the amount entering the living epidermis over time. In vivo findings showed an interesting enhancement drug permeation when positively charged liposomes were used, giving rise to a more pronounced erythematous effect.

8.2.3 Coating with Charged Polymers

The lipid lamellae of the SC contain a high ratio of negatively charged lipids that are expected to interact with cationic liposomes. Transfer of some of the bilayer components of the liposomes to the skin is then possibly induced. Recent studies showed the efficacy of ionic and non-ionic polymers in improving skin compatibility of drug formulations and enhancing the penetration of bioactive compounds (Kadajji and Betageri 2011).

There has been much interest in studying the structures resulting from the self-assembling of liposomes with natural or synthetic charged polyions, because of their possible applications in the enhancement of drug delivery across the skin. Several authors (Mateescu et al. 1999; Nguyen and Shklovskii 2001) have postulated that, in the case of charged liposomes, the short-range attractive interaction (and hence the aggregation) is promoted by the addition of adsorbing oppositely charged polyions. This peculiar mechanism produces an interesting and counterintuitive phenomenon that has been termed "charge inversion." This effect occurs when more polyions than necessary to the complete charge neutralization adsorb at the surface and the sign of the overall surface charge changes. This is a manifestation of the counterion condensation on the polymer backbone, resulting in an increase in a number of condensed counterions (Dobrynin et al. 2000). When the charge of the polyion coating is almost completely neutralized by the liposome surface charge ("isoelectrical point" or charge neutralization), the short-range attractive potential arising from the nonhomogeneous charge distribution promotes the aggregation of complexes.

Several polymers such as carboxymethyl chitin, chitosan (Rengel and Barisic 2002; Guo et al. 2003), poly(vinyl alcohol) (Takeuchi et al. 2000), Eudragit® EPO (Hasanovic et al. 2010), and polyethylene glycol (Jain and Jain 2008; Knudsen et al. 2012a) have been used for preparation of polymer-coated liposomes to enhance the skin permeation of drugs.

Chitin is a linear cationic heteropolymer of randomly distributed 2-acetamido-2-deoxy-β-d-glucose residues with β (1 → 4) linkage. Higher water solubility is expected at an acetylation value lower than 50 %. Many derivatives of chitin are described in the literature with this purpose (Sashiwa and Shigemasa 1999; Park and Park 2001). Among them, carboxymethyl chitin (N-CMC) has been used for skin delivery, serving as a coating material for liposomes (Muzzarelli 1988).

Chitosan is a product derived from *N*-deacetylation of chitin in the presence of hot alkali. The degree of deacetylation and the degree of polymerization (DP), which in turn decides the molecular weight of polymer, are two important

parameters dictating the use of chitosans for various applications.

This polymer seems to be an optimal candidate to be combined with liposomes (González-Rodríguez et al. 2007). Turbidity studies revealed that the coating of DPPC liposomes with chitosan did not significantly modify the main phase transition temperature of DPPC at tested chitosan concentrations (Mady and Darwish 2010). As a cationic biopolymer, it showed the ability to improve skin compatibility of skin formulations, and an enhancing effect on the skin penetration of drugs (Hasanovic et al. 2009). Takeuchi et al. (1996) showed that the chitosan-coated liposomes were formed via ionic interaction between the positively charged chitosan and negatively charged dicetyl phosphate on the surface of the liposomes. By combining chitosan and liposomal characteristics, specific, prolonged, and controlled release may be achieved (Li et al. 2011a).

Chitosan and derivatives are able to enhance the paracellular permeability of hydrophilic and macromolecular drugs by transiently opening the tight junctions in the epidermal barrier (Valenta and Auner 2004). This mechanism can be exerted because at skin pH value (5.5), chitosan is protonated. It can thus interact with anionic components of glycoproteins on the surface of the epidermal cells and with fixed negative charges in the interior of the tight junction, which triggers the opening of the tight junctions, facilitating the transport of hydrophilic compounds (González-Rodríguez et al. 2007).

Chitosan coating resulted in a particle size increase and a more positive zeta potential of liposomes, forming a more stable system, as was recently described by Mady and Darwish (2010). Other studies have focused on the investigation of the effect of chitosan concentration and lipid type (high purity and low purity) on the characteristics of chitosan-coated liposomes and their interactions with the drug. Results showed that polymer bridging caused flocculation at low polymer concentration, whereas at high concentration, the adsorbed chitosan molecule led to steric stabilization. Moreover, leuprolide entrapment efficiency decreased when chitosan was added to liposomes, showing the disturbance of bilayers. In addition, the leakage of leuprolide from low purity liposomes was greater than that from high purity liposomes, because low purity lipids possessed more negative charge and formed thicker adsorptive layer by stronger electrostatic attraction with chitosan (Guo et al. 2003). This disturbing effect may contribute to delay the skin permeation process.

With respect to the control of drug release, chitosan coating has a significant effect on drug release behavior. Appropriate combinations of the liposomal and chitosan characteristics may produce liposomes with specific and prolonged release of drugs, such as doxorubicin (Mady and Darwish 2010), steroid hormones (Biruss and Valenta 2006), acyclovir, and minoxidil (Hasanovic et al. 2010). In addition, the association of chitosan with liposomes is expected to also affect the release rate of glycolic acid from the vesicles by decreasing it; in fact this compound is very small and hydrophilic, and for these reasons it is very difficult to control its diffusion rate from lipidic bilayers.

Chitosan-EDTA was identified as an interesting gelating agent for topical formulations. It was therefore formulated with liposomes as a coating agent. The introduction of EDTA on the backbone of chitosan converts this cationic polymer to an anionic polymer which displays strong mucoadhesive properties, which can be explained by the hydrogen bond formation of its carboxylic acid groups with the mucus gel layer (Bernkop-Schnurch and Krajicek 1998). Biruss and Valenta (2006) suggested that chitosan-EDTA acts by disrupting the intercellular tight junctions (Smith 2003), and it has been proposed as a promising vehicle especially for the skin because of its broad spectrum against bacteria. This antimicrobial activity can be explained on the basis of the mechanism of action, which includes a high binding affinity to magnesium and calcium (Valenta et al. 1998). These bivalent cations are essential components in the outer membrane of bacteria and chitosan-EDTA has a chelating effect on these ions.

In general, PEG is considered the most commonly used polymer for steric stabilization of liposomes, because its hydrophilic structure

introduces a sterical barrier between adjacent bilayer membranes to prevent aggregation (Allen et al. 2002). The effect of PEGylating liposomes for topical application is poorly described in the literature. Besides using PEGylated coating liposomes to enhance stability and permeation properties of low-molecular-weight drugs, such as zidovudine (Jain and Jain 2008) or calcipotriol for the psoriasis treatment (Knudsen et al. 2012a), the use of PEGylated liposomes for dermal application provides the option for active targeting strategies to specific cell types in the skin through conjugation of receptor-specific ligands to the distal ends of the PEG chains (Knudsen et al. 2012b).

8.3 New Applications of the Surface-Charged Vesicles as Skin Permeation Enhancers

The transdermal delivery of biodrugs such as proteins, peptides, and vaccines has been a topic of extensive research in recent years.

Although the charge of the liposome may not influence the penetration efficacy through the SC, the charge may influence the interaction with DNA, thus influencing its penetration. Hong-Yu et al. (1999) developed a formulation for topical skin gene delivery that utilized naked plasmid DNA. The in vivo successes with transfersomes led to their introduction as carriers for noninvasive gene delivery (Kim et al. 2004). In vitro transfection efficiency of plasmid DNA was assessed by the expression of green fluorescent protein (GFP). It was also tested for in vivo transfection efficiency and its retention time within the organs, by applying the complexes on hair-removed dorsal skin of mice, noninvasively. It was found that genes were transported into several organs for 6 days once applied on intact skin, suggesting promising properties for noninvasive gene delivery.

In spite of the literature concerning the formulation of cationic liposomes for topical pDNA delivery (Babiuk et al. 2002; Kim et al. 2004; Akita et al. 2009), the interest for cutaneous siRNA therapy is considerable (Geusens et al. 2009). Several penetration-enhancing techniques in combination with liposomal formulations have been described (Tran et al. 2004). Geusens et al. (2010) developed a new lipid-based nanosome that enables the effective delivery of siRNA into the human skin. The major finding is that ultraflexible siRNA-containing liposomes, prepared using DOTAP, cholesterol, sodium cholate, and ethanol (SECosomes), penetrate into the epidermis of freshly excised intact human skin and are able to enter the keratinocytes. On the other hand, Knudsen et al. designed PEGylated liposomal formulations for topical delivery of calcipotriol, demonstrating that the colloidal stability of liposomes can be increased by PEG.

The transcutaneous route is particularly attractive for immunization, because the skin is highly accessible and has unique immunological characteristics (Li et al. 2011b). The presence of professional antigen-presenting cells (APCs) in the epidermis and dermis mediates the immune response following cutaneous immunization (Kupper and Fuhlbrigge 2004). However, the SC acts as a barrier for diffusion and thereby forms a major obstacle to transcutaneous immunization (TCI), e.g., vaccination through intact or pretreated skin. Currently, the main challenges for cutaneous immunization are to enhance the transport of antigens across the skin barrier and to improve the immunogenicity of topically applied subunit vaccines (Bal et al. 2010). Among them, cationic liposomes may allow them to be widely used as noninvasive delivery agents for vaccine antigens into the skin (Babiuk et al. 2000). When formulating vaccine antigens with liposomes, it is critical to determine the compatibility of the antigen with the liposome. A major concern with the use of liposomal delivery systems for vaccine antigens is toxicity to cells of the immune system. Cationic lipids are highly toxic to phagocytic cells such as macrophages (Filion and Phillips 1997). This toxicity may be due to destabilization of the lysosomal membranes by the cationic lipids (Wattiaux et al. 1997). Transcutaneous immunization offers advantages as effective, safe, and rapid delivery of drugs at the preferred site.

The use of Transfersome™ to formulate antigens in transcutaneous immunization has also been reported in a few studies. Formulations prepared with soybean phosphatidylcholine (PC), Sorbitan monooleate (Span® 80, Croda International PLC), and ethanol were loaded with hepatitis B surface antigen (HBsAg). A similar immune response was induced as compared to those obtained by IM injection of the same dose of alum-adsorbed HBsAg (Mishra et al. 2006a). In contrast, elastic cationic liposomes made of PC, Span® 80, and DOTAP did not improve the immune response when loaded with diphtheria toxoid. Transcutaneous immunization of all formulations resulted in substantial antibody titers only if microneedle pretreatment was applied (Ding et al. 2011).

The skin is an attractive target tissue for somatic genes. In this sense, the promoter elements of tissue-specific genes, including keratin genes, have been identified and can be used to target the expression of genes delivered into the skin. In addition to treating skin disorders, cutaneous gene delivery can be used to express gene products with systemic effects.

A promising study has recently been developed using asiaticoside-loaded ultradeformable vesicles as suitable carriers for the pharmaceutical and cosmetic application of this natural agent in the treatment of diseases characterized by impairment of collagen biosynthesis in cases of both the presence and absence of cutaneous integrity (Paolino et al. 2012).

Conclusions

The addition of charged agents to the overall structure of the lipid vesicles constitutes an interesting strategy to improve the stability and skin permeation properties of drugs.

It is necessary to know the physicochemical properties of ionization of drugs incorporated into vesicles formulated with charged-lipid components, as this would affect the retention of the drug in the SC of the skin or the access to the viable epidermis. Drug solubility and ionization ratio in the pH conditions of the aqueous compartment of vesicles can alter the surface charge and the electrostatic and hydrophobic interactions with the SC.

The use of charged surfactants to increase the deformability of vesicles has an improving effect on the permeation flux, being favored when lipophilic non-ionized drugs are entrapped in the vesicles.

Recent studies showed the efficacy of ionic and non-ionic polymers in improving skin compatibility of drug formulations and enhancing the penetration of bioactive compounds. By combining polymer properties and liposomal characteristics, specific, prolonged, and controlled release may be achieved.

Future advances about the use of surface charge vesicles have been developed. Interesting results have been obtained in the use of the skin as an attractive target tissue for somatic genes. In this sense, although the charge of the liposome may not influence the penetration efficacy through the SC, the charge may influence the interaction with DNA. Also, recent studies have demonstrated that transcutaneous route is particularly attractive for immunization, employing charged modified vesicles to transport antigens across the skin barrier and to improve the immunogenicity of topically applied subunit vaccines.

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9 Ultradeformable Vesicles as Skin Drug Delivery Systems: Mechanisms of Action

Gamal M. El Maghraby

Contents

9.1 Introduction

Early 1980s saw the introduction of lipid vesicles into the field of skin drug delivery. Mezei and Gulasekharam (1980) were the first to report an improved skin deposition and reduced side effects after application of liposomes compared to traditional formulation. This finding was further confirmed on subsequent studies even after incorporation of these liposomes in a gel dosage form (Mezei and Gulasekharam 1982). Following the same line, Weiner and coworkers employed a cutaneous herpes simplex virus guinea pig model and showed superior topical delivery of interferon from liposomes compared to a water-in-oil emulsion or aqueous solution (Weiner et al. 1989). The superiority of liposomes was attributed to increased deposition of interferon into stratum corneum and deeper skin strata with no drug detected in the receptor (Egbaria et al. 1990). The potential of liposomes to enhance local anaesthesia after topical application to intact skin was reported by many investigators. Early investigations utilized the pin-prick assay and recorded prolonged anaesthesia from a tetracaine vesicular formulation with the cream formulation being ineffective (Gesztes and Mezei 1988). Prolonged anaesthetic effect was also reported after topical application of liposome-encapsulated lidocaine compared with a conventional cream (Foldvari et al. 1990). These findings reflect greater drug input into the viable epidermal region, but the results were considered as indication for improved skin accumulation. This consideration was supported by recording higher skin deposition after application of liposomes compared to conventional ointment. In a subsequent in vitro study using human skin, topical application of liposomal tetracaine increased both drug permeation through and deposition into skin compared

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to an ointment containing the same drug concentration (Foldvari 1994). Few studies of the early investigations in this field reported enhanced transdermal delivery from standard liposomes. For example, liposomes were shown to deliver higher amounts of lipophilic drugs like progesterone and hydrocortisone across hairless mice skin compared to aqueous solution (Ganesan et al. 1984). A more positive finding was reported for liposome-encapsulated monoclonal antibodies which were distributed rapidly into the deep cutaneous regions of pig skin with a significantly higher percutaneous absorption compared to aqueous solution (Artman et al. 1990a, b).

While researchers were reporting mainly localized or rarely transdermal effects of liposomes, Cevc and Blume (1992) opened another line in which certain types of lipid vesicles (*Transfersomes*^{TM}) can penetrate intact to the deep layers of the skin and may progress far enough to reach the systemic circulation. The performance of these vesicles depends on the method of application with occlusive application being detrimental to their action. These vesicles were given other terminology such as ultradeformable vesicles (El Maghraby et al. 2006, 2008). The proceeding sections will provide a brief overview on the mechanisms of action of liposomes as skin drug delivery system with highlight on future perspectives. For more details, the readers are referred to detailed reviews covering this area (El Maghraby et al. 2008; El Maghraby and Williams 2009).

9.2 Types of Lipid Vesicles

The literature reports employed different terminology to describe lipid vesicles as skin drug delivery systems. Theses terms were used to describe vesicles which are morphologically similar but differ in composition and/or in function (El Maghraby et al. 2008). The terms include *liposomes* as the prototype and are defined as simple vesicles in which one or more lipid bilayers entrap an aqueous volume. They are formed mainly of phospholipids with or without cholesterol and may contain a charging agent. Alteration of the vesicle components produces vesicles with different functions. *Stratum corneum lipid liposomes* (*SCLL*) are vesicles made of skin lipids at a composition similar to the lipid composition of the stratum corneum. *Transfersomes*^{TM} are morphologically similar to liposomes, but they differ in function as they were claimed to traverse intact skin carrying therapeutic amounts of drugs into the systemic circulation (Cevc and Blume 1992). They were described as highly deformable and were described by other authors as *ultradeformable* vesicles (El Maghraby et al. 1999). Phospholipids are the main components of such vesicles with an additional surfactant being included as an edge activator to increase the elasticity of the vesicular membrane and increase the vesicle deformability (Cevc et al. 1995; El Maghraby et al. 2000a). *Flexosomes* are similar to *Transfersomes*^{TM} as they also contain a surfactant; however, they include also a surface charging agent with positively charged ones being superior. *Ethosomes* are phospholipid vesicles, in which ethanol was included in the vesicle components to increase the elasticity. *Niosomes* are structurally similar to liposomes, but comprise surfactants as the main component with cholesterol and may include small proportions of phospholipids. These are more stable and of low cost compared to liposomes with elastic analogues being available as well (Honeywell-Nguyen et al. 2004; Honeywell-Nguyen and Bouwstra 2005). *Vesosomes* are fusogenic vesicular systems which are comprised of a fusogenic phospholipid (dioleoyl phosphatidylethanolamine, DOPE) with phosphatidylcholine (PC) and a positively charged charging agent (dioleoyl trimethylammonium propane, DOTAP). The formulated fusogenic vesicles are incorporated into the interdigitated lipid bilayer of dipalmitoylphosphatidylcholine (DPPC) and cholesterol to provide vesicles within a vesicle (Mishra et al. 2006).

9.3 Mechanisms of Enhanced Skin Delivery from Ultradeformable Vesicles

The mechanisms of vesicular skin delivery have been generally reviewed with different mechanisms being suggested (El Maghraby et al. 2006, 2008; El Maghraby and Williams 2009). This section will concentrate on mechanisms of ultradeformable vesicles and their relation to the method of application. The proposed mechanisms are shown in Fig. 9.1, which reveals four possible mechanisms. These are summarized below.

9.3.1 Penetration-Enhancing Mechanism

This mechanism is based on the possibility that vesicle components can enter the skin as monomers perturbing the packing characteristics of the stratum corneum lipids. This will open the way for drug permeation into and through the stratum corneum (Fig. 9.1a). This mechanism was verified by investigating the transdermal delivery of estradiol from various liposomes and ultradeformable vesicles. The effect of skin pretreatment with placebo (drug-free) vesicular formulation on estradiol skin delivery from saturated aqueous solution was also studied and compared to the

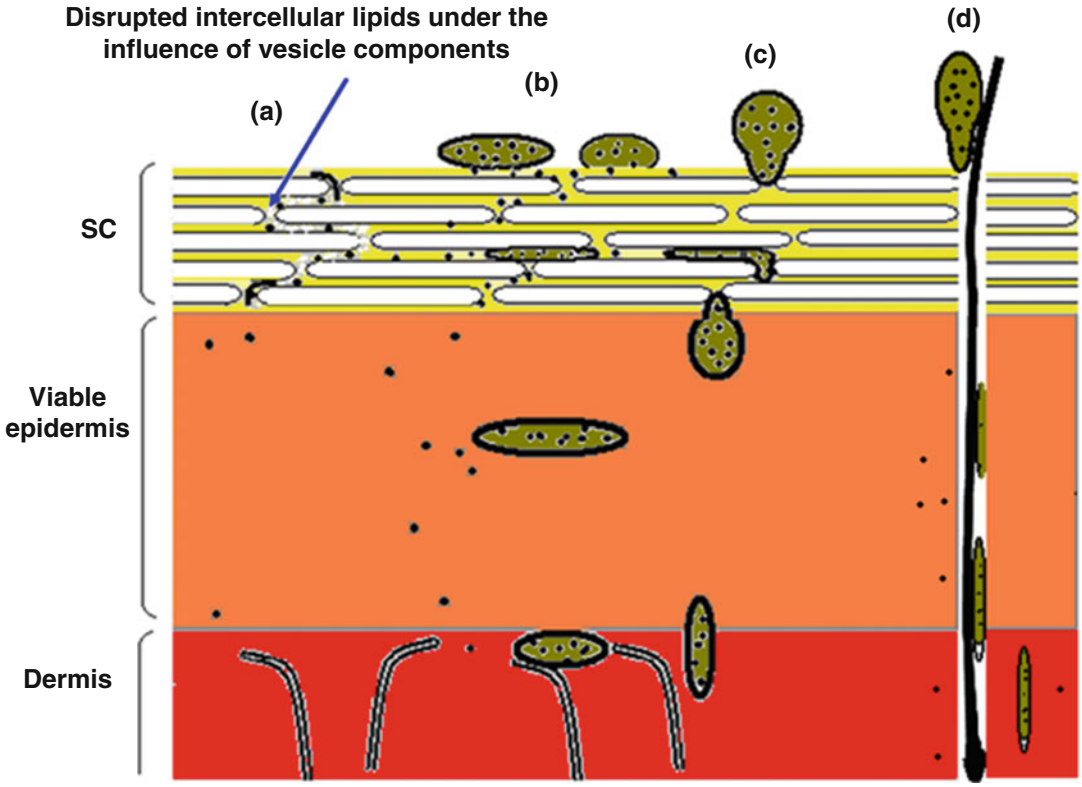

Fig. 9.1 Diagrammatic representation of the skin showing the possible mechanisms of vesicular skin drug delivery. (**a**) Penetration-enhancing effect, (**b**) vesicle adsorption, adhesion and fusion to skin, (**c**) intact vesicle penetration through intact skin and (**d**) vesicle penetration into and through the hair follicle (not to scale) (Modified from El Maghraby and Williams (2009))

delivery of the drug from medicated (drug loaded) vesicles (El Maghraby et al. 1999). The tested systems included vesicles with different membrane elasticity. The ultradeformable formulations included phosphatidylcholine (PC) with sodium cholate, sorbitan monooleate (Span 80®, Sigma Chemical Company, USA) or polyoxyethylene sorbitan monooleate (Tween 80®, Sigma Chemical Company, USA). The standard liposomes were comprised of pure PC (nonrigid liposomes), PC with cholesterol (membrane-stabilized liposomes) and DPPC (rigid liposomes). The results indicated a possible penetration-enhancing effect only for liposomes made of pure PC. This was suggested after recording a fourfold increase in transdermal estradiol flux after pretreatment with empty vesicles. However, comparing enhancing effect of PC vesicles (fourfold) with the relative flux obtained after application of estradiol loaded the same liposomes (eightfold), and it can be concluded that enhancement is not the main mechanism operating. For the membrane-stabilized and rigid liposomes and ultradeformable vesicles, no significant enhancement was recorded after skin pretreatment with placebo formulation delivering significant amounts into and through human epidermis (El Maghraby et al. 1999). This process was further verified by researching the importance of vesicular structure for transdermal delivery (El Maghraby et al. 2000b). This was achieved by comparing estradiol skin delivery from ultradeformable vesicles with that obtained from propylene glycol solutions containing the same vesicular components. The results revealed the importance of the vesicular structure. These studies excluded any important role for the penetration-enhancing mechanism in enhanced skin delivery of drugs from ultradeformable vesicles.

9.3.2 Vesicle Adsorption to and/or Fusion with the Stratum Corneum

According to this hypothesis, vesicles are at least adsorbed to or even fused with the stratum corneum (see Fig. 9.1b). This should at least provide intimate contact with the skin filling the wrinkles with subsequent direct vesicle – skin transfer of drug. This process should improve the uptake of the drug by the stratum corneum. Further, this process was probed by monitoring the uptake of the drug after dipping human stratum corneum into ultradeformable vesicular formulations or aqueous drug solution for short time (El Maghraby et al. 1999). The results revealed significantly higher uptake of estradiol from the vesicular formulations compared to drug solution. These results provide direct explanation for increased skin deposition of drugs from lipid vesicles and imply increased drug portioning into the stratum corneum. Taking into consideration the fact that drug partitioning into the upper skin layers is the first step in drug transfer across the skin, improved drug uptake can be taken as an indication for enhanced transdermal drug delivery from ultradeformable vesicles. This study suggested a possible role of drug adsorption to and/or fusion with the skin surface in transdermal drug delivery from these nanoaggregates. The concept of interaction of lipid vesicles with human skin has been reviewed earlier, and it was suggested that vesicles can be taken into the skin before mixing with intercellular lipids of the SC-forming unit membrane structures (Schaller and Korting 1996). The processes of adhesion onto the skin surface and fusion or mixing with the lipid matrix of stratum corneum have been suggested for liposome lipids (Kirjavainen et al. 1996).

9.3.3 Intact Vesicular Skin-Penetration Mechanism

This mechanism (see Fig. 9.1c) suggests that the intact vesicles can penetrate into and travel through skin strata and *may* go deep enough to reach the systemic circulation. The concept of intact vesicular skin penetration was suggested for traditional liposomes, but evidence was given only for penetration into and not through the skin as evidenced by improved skin deposition of drugs (Mezei and Gulasekharam 1980, 1982). This hypothesis that vesicles could penetrate intact through the skin was born with the first

report on liposomes as skin drug delivery systems (Mezei and Gulasekharam 1980, 1982). This process was researched further after application of traditional liposomes loaded with an electron dense marker to guinea pigs. The electron micrographs revealed the presence of intact liposomes in the dermis. This was taken as an indication for liposomes' penetration through the epidermis (Foldvari et al. 1990). However, negative findings were reported against the hypothesis that traditional liposomes could penetrate even through the skin. For example, Korting and coworkers (1990) concluded that liposomes can penetrate diseased skin with its ruptured SC (as in eczema) but cannot invade skin with hyperkeratosis, as in psoriasis (Korting et al. 1990). Furthermore, more recent studies revealed no evidence of intact carrier penetration into the skin after application of traditional liposomes of dimyristoylphosphatidylcholine (DMPC) or soybean PC (Korting et al. 1995; Zellmer et al. 1995). The first group applied standard PC liposomes to human epidermis reconstituted in vitro. Electron microscopy showed alterations in the morphology of epidermis with shrunken lipid droplets being formed between the corneocytes (Korting et al. 1995). The second group applied DMPC to human skin before performing confocal laser scanning microscopic and differential scanning calorimetric (DSC) investigations. The microscopy revealed no evidence of intact liposome skin penetration, but the DSC suggested that the lipid can penetrate and change the enthalpy of the lipid-related transitions of the SC (Zellmer et al. 1995).

The introduction of *Transfersomes™* into the field of dermal/transdermal drug delivery was associated with the claim of penetration of intact vesicles through the skin, with the nanoaggregates going deep enough to be absorbed by the systemic circulation (Cevc and Blume 1992). The concentration of water varies across the skin strata with the concentration of water increasing as we move from the skin surface to deep strata. At the SC, the water concentration is less than 15 %, but increases deeper into the skin where it reaches a five times higher value at the basal skin layers (Warner et al. 1988). This creates a transdermal hydration gradient which was said to produce a force sufficient to drive the ultradeformable lipid vesicles (*Transfersomes™*) into and through the intact skin. *Transfersomes™* are characterized by xerophobia (tendency to avoid dry surrounding) which causes the vesicles to resist dehydration at the skin surface by moving into the skin with the local transdermal hydration gradient (Cevc and Blume 1992; Cevc et al. 1995). The transdermal hydration gradient was considered sufficient to drive vesicles through the skin due to the high deformability of such vesicles. The vesicles were thus given the name "ultradeformable vesicles" (El Maghraby et al. 2000a, 2001a, b; Cevc et al. 2002). Based on this hypothesis, ultradeformable vesicles need to be applied under nonocclusive conditions as occlusion is believed to abolish the driving force for the skin penetration of vesicles (Cevc and Blume 1992). Surprisingly, the same group applied the local anaesthetic lidocaine entrapped in *Transfersomes™* under occlusion applying a watertight wrapping for 25 min over the applied formulation (Planas et al. 1992). This hypothesis required verification with respect to the possibility of intact vesicles' skin penetration and the need for open application.

To test the intact vesicles' skin penetration process, the effect of vesicle size on transdermal drug delivery was investigated, assuming that this vesicle penetration into and through the skin is a function of the vesicle size. Thus, the permeation of estradiol from large multilamellar vesicles (LMLVs, at least 557 nm in diameter) was compared with that obtained from small unilamellar vesicles (SUVs) of a mean size of 124–138 nm (El Maghraby et al. 1999). The size of the selected SUVs was less than the maximum size reported to enter the skin, and the minimum size of LMLVs is above the maximum volume which can invade the skin (Cevc et al. 1995). The results revealed no significant differences between LMLVs and SUVs with respect to transdermal delivery of estradiol. This finding suggests that intact vesicles do not permeate *through* human epidermal membrane in vitro, based on the assumption that permeation would be a function of carrier diameter. However, it is important to

emphasize that tested LMLVs were prepared using simple mechanical shaking, a technique which produces heterogenous population of vesicles with small- and large-size values being available. This can weaken the conclusion of the study. In a more recent study, small vesicles improved both drug deposition into deeper strata and penetration through the skin, with larger vesicles improving deposition only (Valenta and Janisch 2003; Verma et al. 2003).

Another approach to measure the ability of intact vesicles to enter into and through the narrow tortuous pathway of the skin is to measure the ability of such vesicles to pass through a narrow orifice without being changed in size. This was believed to provide a measure for the ultradeformability of vesicles. Elastic vesicles were shown to penetrate a microporous filter with a pore diameter three times smaller than their own span. However, these vesicles were able to show only improved skin deposition of drug (Trotta et al. 2002). This finding may support the possibility that vesicles can penetrate into but not through the skin. Similar studies revealed that the size of ultradeformable vesicles was unchanged after extrusion through semipermeable membrane barriers (Cevc and Gebauer 2003). These ultradeformable vesicles were detected in mice blood after topical application of fluorescent-labelled vesicles. It is interesting to note that the size of the detected vesicles was similar to that of the starting liposome dispersion. This was taken as a clear evidence for vesicle invasion into and through the skin (Cevc et al. 2002; Cevc and Gebauer 2003). Fast delivery of intact elastic vesicles into the stratum corneum was recorded after their topical application onto human skin (Honeywell-Nguyen et al. 2003). This invasion is thought to be via channel-like regions in the stratum corneum (SC) with nonocclusive application being superior to occlusive application (Honeywell-Nguyen et al. 2003). El Maghraby and Williams (2009) considered the above findings as indications for the possibility that some intact vesicles may penetrate into healthy skin.

To investigate the possibility that the transdermal hydration gradient provides the driving force for intact vesicles' skin penetration, occlusive versus open application was monitored. For example, radiolabelled lipid components were used to trace the fate of ultradeformable vesicles after occlusive and nonocclusive application (Cevc and Blume 1992). After occlusive application, 25 % of the dose of the drug was detected in the stratum corneum, a very few percents in the deeper layers of the epidermis, but most of the dose was recovered from the skin surface. However, with open application, 30 % of the dose was found in the subdermis, and up to 6–8 % was detected in the blood. This demonstrated the importance of an open application of these ultradeformable nanoaggregates, although a deviation from this protocol can be found by the same group (Planas et al. 1992), where an improved anaesthetic effect was reported after occluded treatment with anaesthetic ultradeformable vesicles. In a more recent investigation, the transdermal delivery of estradiol from ultradeformable vesicles was studied after open application to partially hydrated skin. This was compared to the drug delivery from the same vesicles after occluded application to fully hydrated skin. Partial hydration of human skin was achieved by the so-called open hydration protocol in which the epidermal membrane was hydrated from viable epidermal side with the stratum corneal surface being left open to mimic the in vivo conditions (El Maghraby et al. 2001b). In this study, ultradeformable formulations comprising phosphatidylcholine (PC) with sodium cholate (UD1), PC with sorbitan monooleate (Span 80®, Sigma Chemical Company, USA) (UD2) and PC with polyoxyethylene sorbitan monooleate (Tween 80®, Sigma Chemical Company, USA) (UD3) were used. Saturated aqueous solution of the drug was used as control. The transdermal flux values were calculated relative to that of the control. The results are shown in Fig. 9.2. The results revealed significant reduction in the transdermal delivering efficiency after occlusive application compared with open application (taking the relative transepidermal flux as a measure for this efficiency). The delivering efficiency was reduced by 62, 47 and 50 % after occlusive application compared with open application of sodium cholate-containing (UD1), Span®-containing

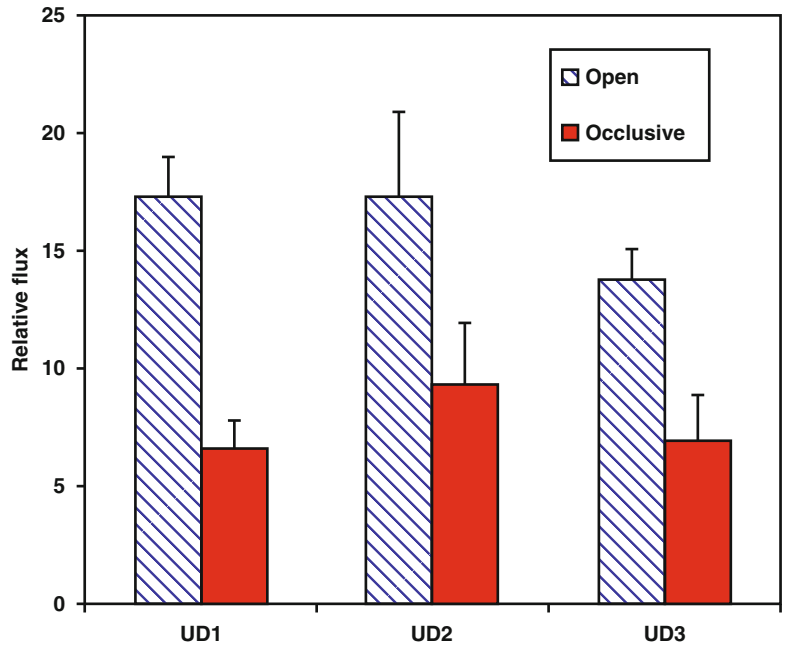

Fig. 9.2 The relative transdermal flux of estradiol, obtained after open and occlusive application of ultradeformable vesicles to human epidermal membrane. The relative flux was calculated with reference to the flux obtained from saturated aqueous drug solution applied similarly to skin samples obtained from the same donors. UD1, UD2 and UD3 are ultradeformable vesicles based on PC and sodium cholate or Span 80 or Tween 80, respectively (Produced using the data published by El Maghraby et al. (1999))

(UD2) and Tween®-containing (UD3) ultradeformable vesicles, respectively. These results highlighted the need for the open application protocol and provide a support for the hydration gradient theory (El Maghraby et al. 2001b).

9.3.4 Transappendageal Penetration

Occlusive application and full skin hydration were shown to inhibit or at least reduce the transdermal drug-delivering ability of ultradeformable liposomes. This effect was attributed to the inhibition of the transdermal hydration gradient, which is thought to provide the driving force for intact vesicles' skin penetration (Cevc and Blume 1992; Cevc et al. 1995). This was considered cautiously with alternative explanation suggested. This alternative relies on the fact that overhydration of the skin can swell the corneocytes and thus close or at least narrow the appendageal pathway that may play a role in vesicular skin delivery (see Fig. 9.1d). To explore this possibility, a new in vitro technique employing human abdominal skin was developed. The study probed estradiol vesicular delivery through epidermis. This was compared to the delivery through a sandwich of stratum corneum (SC) and epidermis, where the additional SC formed a top layer and closed the openings of skin appendages. This development was based on the fact that the orifices of shunts occupy only about 0.1 % of the total skin surface area. Accordingly, there will be a negligible chance that shunts in the two membranes would superimpose. It was thus assumed that the top layer of SC would block most of the shunts available in the bottom membrane (El Maghraby et al. 2001b). The results of this investigation indicated a minor role for the shunt route in transdermal delivery of estradiol from lipid vesicles. This conclusion supports earlier findings which excluded vesicular delivery through shunts on the basis of the lack of significant differences between different animals or humans with diverse densities of hair follicles, with respect to the transdermal vesicular delivery of insulin (Cevc et al. 1998). In a more recent study, the transfollicular delivery from liposomes was enhanced only after combination with iontophoresis (Han et al. 2004). Another research group employed hydrophilic (carboxyfluorescein) and lipophilic (curcumin) fluorescent dyes as model drugs to investigate the follicular delivery of

drugs from liposomes using porcine skin. The penetration depth of the dyes into the hair follicle was measured by laser scanning microscopy in histological sections. The liposomes showed a higher penetration depth compared to the standard formulation. Amphoteric and cationic liposomes reached an average relative penetration depth of approximately 70 % of the full hair follicle length (Jung et al. 2006).

It seems that the shunt routes play no major role in vesicular *transdermal* delivery. However, vesicle penetration into, but not necessarily through, hair follicles (i.e. targeting) cannot be excluded and is clearly demonstrated by numerous literature reports (El Maghraby et al. 2006; El Maghraby and Williams 2009). It was suggested that the particulate delivery systems aggregate and remain in hair follicle openings, and their penetration along the follicular duct occurs in a size-dependent manner, which has led to advanced concepts of targeted drug delivery of bioactive compounds. However, pilot human studies are necessary before going any further in this field (Knorr et al. 2009).

9.4 Conclusion and Future Perspectives

The detailed review of the mechanisms of enhanced transdermal drug delivery from lipid vesicles revealed that a combination of mechanisms is the most probable mechanism. The most important point to be considered is that most of the proposed mechanisms require the fluidity of vesicular formulations (i.e. vesicles are dispersed in liquid media). This may complicate the process of scaling up and development of new dosage form based on these nanoaggregates. This complication comes from the fact that fluid vesicular systems are less stable and tend to loose the entrapped drug. This instability is expected to increase with increased elasticity and deformability of the vesicular membrane. Accordingly, extensive research is required to develop stable topical formulations while maintaining the transdermal drug delivery power. On the light of this, vesicle pro-concentrates have been prepared and investigated. This technique was initially applied for niosomes and called proniosomes which contain all the vesicular components with ethanol and only traces of water. These systems are gel-like preparations and are believed to generate vesicles upon application to the skin due to their mixing with skin secretions such as the sweat, before delivering their payload into and through the skin. Now pro-ultradeformable vesicles are being developed and tested, and the coming years will show to what extent scientist will succeed in this approach.

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Niosomes and Proniosomes for Enhanced Skin Delivery

10

Rita Muzzalupo

Contents

10.1 Skin and Penetration Enhancers

The skin is very effective as a selective penetration barrier. Percutaneous absorption involves the passage of the drug molecule from the skin surface into the stratum corneum under the influence of a concentration gradient and its subsequent diffusion through the stratum corneum and underlying epidermis, through the dermis, and into the blood circulation. The skin behaves as a passive barrier to the penetrant molecule. The stratum corneum provides the greatest resistance to penetration, and it is the rate-limiting step in percutaneous absorption (Barry 2001; Inayat and Mallikarjuna 2009).

An approach commonly researched, for promoting permeation through the skin of poorly penetrating drug molecules, is the formulation of a suitable delivery vehicle or the incorporation of a chemical enhancer into dermal and transdermal delivery systems. Penetration enhancers are substances that facilitate the absorption of penetrants through the skin by temporary diminishing the impermeability of the skin. Ideally, these materials should be pharmacologically inert, nontoxic, nonirritating, nonallergenic, compatible with the drug and excipients, odorless, tasteless, colorless, inexpensive, and have good solvent properties (Williams and Barry 2004). The enhancer should not lead to the loss of body fluids, electrolytes, and other endogenous materials, and the skin

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should immediately regain its barrier properties on its removal (Davis and Hadgraft 1991; Pfister et al. 1990). Alternatively, physical mechanisms such as iontophoresis and phonophoresis can be used to promote the diffusion of certain classes of drugs (Venuganti and Perumal 2009).

The primary role of the stratum corneum is to provide a substantial diffusional barrier and thereby protect the body from the ingress of xenobiotics. The dermal barrier of the body is now known to be a complex, dynamic, biochemical environment that responds to ambient conditions to maximize the protective barrier. Diffusional resistance is known to reside in the stratum corneum, and it is constituted by a complex interaction, principally of lipid and protein components, which create fairly distinct hydrophilic and lipophilic penetration pathways. The increase in the understanding of the function of the stratum corneum in recent years has resulted in a diverse range of compounds being tested for their ability to facilitate improved permeation of coadministered drugs through the skin (Vyas and Khar 2002). Penetration-enhancing effects, resulting from structural alterations of the stratum corneum barrier, manifest themselves in an increase of the drug diffusion coefficient and/or of the drug solubility in the barrier. The quantification of enhancing effects on drug penetration is possible either by the direct determination of the drug fluxes or by an indirect determination through the measurement of the pharmacodynamic response.

Percutaneous applied drug preparations for local or systemic therapy still have limited efficacy.

The transdermal delivery of systemically acting drugs may cause problems. The number of drugs that lead to plasma levels in the therapeutic range after transdermal application is small. This is limited due to the significant barrier to penetration across the skin, consequently even for readily penetrating drugs, such as nitroglycerin, the daily dose of drug that can be delivered from a transdermal patch is not more than a few milligrams (Guy and Hadgraft 1985). The penetration rate of drugs from transdermal systems does not really seem to be regulated by the drug release rate from the transdermal system (Chien et al. 1983; Tojo et al. 1986): the drug penetration across the stratum corneum is the penetration rate-limiting step. The reason for these problems is the distinct barrier function of the stratum corneum. The drug penetration rate and the permeability of the barrier for the drug increase primarily with increasing drug's partition coefficients between stratum corneum and vehicle or between octanol and water and with decreasing relative molecular weight (Flynn 1990; Hagedorn-Leweke and Lippold 1995; Le and Lippold 1995). High skin permeability can be expected, in particular, for small molecules with sufficient affinity to the stratum corneum.

The final aim of transdermal drug delivery is to ensure that compounds are delivered, preferably at a specific rate, to the systemic circulation. Penetration of the drug to the dermal vasculature follows exposure of the skin to a dosage form from which the active must partition, followed by diffusion of the compound through the external strata to the dermis. Partitioning of the drug from the dosage form to the skin is highly dependent on the relative solubility of the drug in the components of the delivery system and in the skin. Thus, the composition of the vehicle may markedly influence the degree of penetration of the drug (Guy and Hadgraft 1989). Partitioning is governed to a large extent by the thermodynamic activity of the drug in the vehicle, and this is, therefore, of major importance in controlling the degree of penetration of any compound (Chien 1991).

Since diffusion of drugs across the skin is a passive process, compounds with low solubility and low affinity for the hydrophilic and lipophilic components of the SC would, theoretically, partition at a slow rate. These difficulties may be overcome by addition of a chemical adjunct to the delivery system that would promote drug partitioning into the SC. Furthermore, penetration enhancer chemicals added to topical vehicles usually also partition into the SC and affect the intrinsic diffusional barrier properties of this structure. Other factors that require consideration, when penetration enhancers are included into formulations, are the effects that these

chemicals may have on the solubility of the drug in the delivery vehicle, influencing the diffusional gradient, or the possible effects that the chemical may have on the state of hydration of the diffusional barrier (Vyas and Khar 2011).

Different classes of compounds have been tested for their enhancer action (Sinha and Kaur 2000), and different approaches to enhance drug penetration which include the use of enzymes, natural oils, phospholipid micelles, liposomes (Sinico et al. 2005), niosomes (Tavano et al. 2011), polymers, lyotropic liquid crystals (Muzzalupo et al. 2010), and surfactants (Bettley 1965).

Carrier-based percutaneous drug delivery systems are represented in Fig. 10.1.

Surfactants contribute to the overall penetration enhancement of compounds primarily by adsorption at interfaces, by interacting with biological membranes, and by alteration of the barrier function of the SC, as result of reversible lipid modification (Kushla et al. 1993). Nonionic surfactants are used widely in pharmaceuticals to increase stability, solubility, and permeation of drugs.

Among these strategies, special formulation approaches based mainly on the use of surfactant solutions or vesicles (niosomes and proniosomes) are the most promising (Choi and Maibach 2005).

There is a direct contact of niosome/proniosome formulations with the skin after their application, so it is better to discuss the potential interactions between the skin and vesicles formed in niosome/proniosome formulations. As known, niosomes and proniosomes are vesicles composed of nonionic surfactants. So it is advisable to study the interactions between nonionic surfactants and the skin. Nonionic surfactants are used widely in pharmaceuticals to increase stability, solubility, and permeation of drugs. There is a strong indication that the degree of interaction between vesicles and skin mainly depends on physicochemical properties of the surfactant molecules which the niosomes or proniosomes are composed of.

10.2 Niosomes as Carriers in Percutaneous Drug Delivery

Colloidal vesicular carriers such as niosomes have been extensively applied in drug delivery systems due to their unique advantages. The formation of vesicular systems based on hydration of a mixture of a single-alkyl chain nonionic surfactant and cholesterol was firstly reported in 1979 (Handjani-Vila

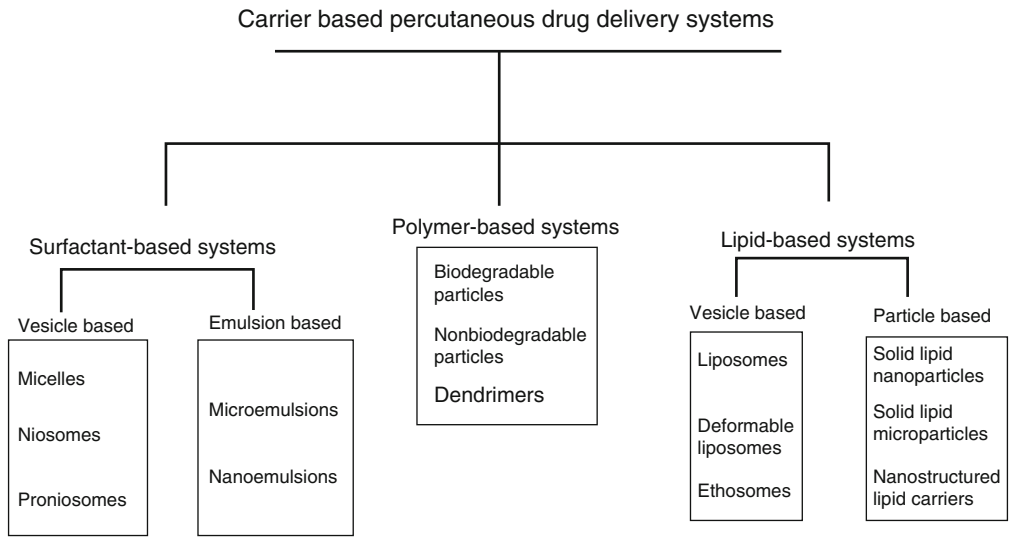

Fig. 10.1 Carriers for percutaneous drug delivery (Adapted from Venuganti and Perumal 2009)

et al. 1979). Niosomes are self-assembled, submicron vesicles composed of nonionic surfactants with closed bilayer structures similar to liposomes. However, they are much more stable and less expensive than liposomes. Niosomes are synthetic microscopic vesicles consisting of an aqueous core enclosed in a bilayer consisting of one or more nonionic surfactants and cholesterol. They are made of biocompatible, biodegradable, nontoxic, nonimmunogenic, and noncarcinogenic agents which form closed spherical structures (self-assembly vesicles) upon hydration. With high resistance to hydrolytic degradation, niosomes are capable of entrapping many kinds of drugs and exhibit greater stability and longer shelf life than liposomes. These vesicles can act as drug reservoirs, and the rate of drug release can be modified by changing their composition (Handjani-Vila et al. 1979; Sankhyan and Pawar 2012; Tojo et al. 1986; Udupa 2004).

These vesicular carriers can encapsulate both hydrophilic drugs (in the aqueous core) and hydrophobic drugs (in the bilayer). Because of their potential to carry a variety of drugs, these vesicles have been widely used in various drug delivery systems, for drug targeting, controlled drug release, and permeation enhancement of drugs (Akhilesh 2011). The hydrophobic part of the surfactant faces toward the core, whereas the hydrophilic groups interface with the surrounding aqueous medium. Niosomes can be constructed by using a variety of surfactants, which possess a hydrophilic head group and a hydrophobic tail. The hydrophobic tail may consist of one or two alkyl or perfluoroalkyl groups, or in some cases, it consists of a single steroidal group. The surfactants with an alkyl chain length from C12–C18 are suitable for the preparation of niosomes. Hydrophilic head groups include glycerol, ethylene oxide, polyhydroxy groups, crown ethers, sugars, and amino acids (Lohumi et al. 2012; Sahin 2007). The most frequently used nonionic surfactants include ester of sorbitan (sorbitans are also known as "Spans") and polyoxyethylene alkyl ether (C_nEO_m, Brij®) surfactants. Cholesterol and their derivatives are included in niosomes besides surfactants, usually in a 1:1 molar ratio, as steric stabilizers to prevent aggregation (Florence 1993; Vyas and Khar 2011). Cholesterol also prevents the phase transition of niosomes from the gel to the liquid state and thereby reduces drug leakage from niosomes. The stability of niosomes can be further improved by the addition of charged molecules such as dicetyl phosphate, which prevents aggregation by charge repulsion. Generally, an increase in surfactant/lipid level increases the drug encapsulation efficiency in niosomes (Uchegbu and Vyas 1998). Preparation of niosomes requires some energy in the form of elevated temperature and/or shear. The majority of the methods involve hydration of a mixture of surfactant/lipid at elevated temperature, followed by size reduction using sonication, extrusion, or better high-pressure homogenization. Finally, the removal of unentrapped drug from the vesicles can be accomplished by dialysis, gel filtration, or centrifugation. Niosomes prepared by hydration methods usually are in micron size range. Size reduction by sonication and/or extrusion results in niosomes of 100–200 nm, whereas microfluidizer or high-pressure homogenizer can achieve niosomes of 50–100 nm. The smaller size of niosomes is achieved at the cost of reduced drug loading. Furthermore, the smaller niosomes are relatively more unstable than larger ones and, therefore, require stabilizers, such as cholesterol or fatty alcohols or diacetyl phosphate to prevent aggregation. Both hydrophilic and hydrophobic drug molecules have been encapsulated in niosomes by using either dehydration–rehydration technique or the pH gradient within and outside the niosomes (Uchegbu and Vyas 1998; Vyas and Khar 2011).

Percutaneous delivery defines targeting to the pathological sites within the skin with the least systemic absorption. Drug localization in the skin is of crucial importance in the treatment of dermatological diseases such as skin cancer, psoriasis, alopecia, and acne, where the origin of disease is located in the skin (Brown et al. 2006). Drug administration onto the skin has been used since a long time to deliver drugs to different skin layers as well as to deep regions somewhat

remote from the application site. However, several limitations have been associated with conventional topical preparations, e.g., low percutaneous drug penetration because of the barrier function of the stratum corneum, the outermost layer of the skin (Rubio et al. 2011), or unwanted absorption to the systemic circulation (Dubey et al. 2012). Research studies report nowadays about delivery systems that are able to deliver drugs through the skin (Higaki et al. 2005).

Niosomes have been reported to enhance the residence time of drugs in the stratum corneum and epidermis, while reducing the systemic absorption of the drug and improving penetration of the trapped substances through the skin. In addition, these systems have been reported to decrease side effects of drugs. (Schreier and Bouwstra 1994). They are thought to improve the horny layer properties both by reducing transepidermal water loss and by increasing smoothness via replenishing lost skin lipids (Hofland et al. 1991). Moreover, it has been reported in several studies that compared to conventional dosage forms, vesicular formulations exhibited an enhanced cutaneous drug bioavailability (Manconi et al. 2006; Mura et al. 2007; Srikanth et al. 2010).

Therefore, the main advantages of niosomal systems are as follows (Biju et al. 2006; Indhu et al. 2004; Vyas and Khar 2011):

- They consist of hydrophilic, amphiphilic, and lipophilic moieties together and as a result they can accommodate drug molecules with a wide range of solubilities.
- The vesicles have flexible properties that can be varied by changing, the composition of vesicles, which affects the size, lamellarity, tapped volume, and surface charge.
- As the vesicle dispersion is a water-based vehicle, it provides better patient compliance than oil-based dosage forms.
- By improving oral bioavailability of poorly absorbed drugs, by delaying clearance from the circulation, and by protecting the drug from biological environment, they improve the therapeutic performance of the drug molecules.
- The vesicles may act as a depot, releasing the drug in a controlled manner.
- They are osmotically active, chemically stable, and also increase the stability of the drug entrapped. Oral, parenteral, and topical routes can be used for their administration.
- Biodegradable, biocompatible, and nonimmunogenic surfactants are used in the preparation of niosomes.
- Handling and storage of surfactants require no special conditions.

The drawbacks of niosomes are principally linked to their preparation (Verma et al. 2010):

- The aqueous suspensions of niosomes may have limited shelf life due to fusion and aggregation of vesicles and leaking of entrapped drugs from vesicles.
- The methods of vesicle preparation foreseeing the steps, such as extrusion and sonication, are time consuming and may require specialized equipment for processing.

The entrapment efficiency of the drug, the rate of drug release, and the size of vesicles are dependent on the hydrophilic-lipophilic balance (HLB) value of the surfactant. The entrapment efficiency increases with the increase in the concentration and lipophilicity of the surfactant, while if the HLB value of the surfactant decreases, the mean size is reduced (Biswal et al. 2008; Lawrence et al. 1996; Shahiwala and Misra 2002).

The rate of drug release from niosomes is dependent on the surfactant type and also on the phase-transition temperature of surfactant. For example, the release of carboxyfluorescein from sorbitan monoester (sorbitan molaurate, Span® 20; sorbitan monopalmitate Span® 40; sorbitan monostearate, Span® 60;) niosomes was in the following decreasing order: Span® 20 > Span® 40 > Span® 60, i.e., the release decreased with an increase in alkyl chain length of the surfactant [82]. Their phase transition temperature (from gel to liquid thermodynamic state) increased as the length of the acyl chain increased. Thus, sorbitan monolaurate (Span® 20) with a C9 chain is in liquid thermodynamic

state at room temperature; sorbitan monopalmitate (Span® 40) with a C13 chain has a phase transition temperature of 46–47 °C; sorbitan monostearate (Span® 60) with a C15 chain has a gel to liquid phase transition temperature of 56–58 °C. Vesicles made with these higher molecular weight Span® surfactants are less leaky and more stable to osmotic gradients. Niosomes have been shown to penetrate into the skin and enhance the permeation of drugs. Span® niosomes provided significantly higher skin permeation and partitioning of enoxacin than liposomes and the free drug. The action of niosomes as permeation enhancers might predominantly be on the intercellular lipids of SC, raising the fluidity and weakness of the SC. The direct permeation of the vesicles into the viable epidermis and dermis was largely restricted. Niosomes were mainly localized in the SC, but niosomes largely contribute to the rapid permeation of enoxacin across the SC which may be due to the higher diffusion of vesicles with the drug into the SC. It is proposed that niosomes disrupt the membrane properties of the SC as well as that they directly fuse into the upper layer of the skin, thereby enhancing the skin permeation of enoxacin (Fang et al. 2001a). Niosomes dissociate and form loosely bound aggregates, which then penetrate into the deeper skin strata. Furthermore, the high skin penetration of drugs from niosomes has been attributed to the flexibility of niosomes, and this is supported by the fact that a decrease in cholesterol content which leads to higher vesicle flexibility increases the drug penetration through the skin (Vanhal et al. 1996). Moreover, the nonionic surfactant can also modify the intercellular lipid structure in the SC to enhance skin permeability. In addition, adsorption and fusion of niosomes with the skin surface increase the thermodynamic activity of the drug, leading to enhanced drug penetration into the skin (Schreier and Bouwstra 1994). In vitro studies have found that the chain length of alkyl polyoxyethylene in niosomes did not affect the cell proliferation of human keratinocytes, but the ester bond was found to be more toxic than the ether bond in the surfactants (Hofland et al. 1991).

10.3 Percutaneous Applications of Niosomes

Niosomes and niosomal gels are considered to be useful controlled drug delivery systems for the percutaneous route, and many researchers studied their potential as innovative drug delivery systems for this route (Hamishehkar et al. 2013).

5-Fluorouracil (5-FU) is an anticancer drug that showed appropriate antitumoral effect in the topical treatment of lesions associated to squamous cell carcinoma (Gross et al. 2007). Unfortunately, 5-FU shows a low percutaneous permeation, thus reducing its anticancer effectiveness. Therefore, improved percutaneous permeation of 5-FU is an important prerequisite to attain an effective topical therapeutic approach (Gupta et al. 2005; Singh et al. 2005). Paolino et al. designed an innovative niosomal system composed of α,ω-hexadecyl-bis-(1-aza-18-crown-6) (Bola), Span® 80, and cholesterol as a topical carrier system for 5-FU. In this study, the percutaneous permeation of 5-FU through human stratum corneum and epidermal layers demonstrated that bola-niosomes provided an eightfold and fourfold increase of the drug penetration compared to an aqueous drug solution and to a mixture of empty bola-niosomes with an aqueous drug solution, respectively (Paolino et al. 2008). Niosomes loaded with capsaicin were prepared using a particular ratio between surfactants, to obtain systems with a specific HLB value (10, 12, 14) and characterized in terms of particle size, morphology, and their capsaicin entrapment efficiency. They were evaluated in vitro for their percutaneous permeation-enhancing effect. The prepared formulations were compared to microemulsions prepared from the same surfactants in the same ratio, and a higher transdermal delivery of capsaicin was achieved with niosomes (Tavano et al. 2011). Carafa et al. (2002) prepared lidocaine and lidocaine hydrochloride-loaded nonionic surfactant vesicles using Tween® 20 and cholesterol. The ability of the drug to diffuse through a model lipophilic membrane and through mouse skin was studied and compared with classical liposomes and Tween® 20 micelles. Also, dicetylphosphate and N-cetylpyridinium

chloride were used to prepare negatively and positively charged vesicles, respectively, in order to study the effect of vesicle charge on drug encapsulation efficiency. The obtained data provided direct evidence that neutral vesicles, prepared with Tween® 20 and cholesterol, entrapped a higher lidocaine amount, at pH 5.5, than positively and negatively charged vesicles. Ellagic acid may selectively inhibit melanin synthesis only in UV-activated melanocytes. It is a phytochemical substance with potent antioxidative properties which has limited use due to its poor biopharmaceutical properties such as low solubility and low skin permeation ability. Junyaprasert et al. (2012) prepared a niosomal formulation of ellagic acid from the mixture of Span® 60 and Tween® 60 for dermal delivery. Due to the low solubility of ellagic acid, methanol (MeOH), propylene glycol (PG), and polyethylene glycol 400 (PEG 400) were used as solubilizers. Skin permeation and distribution studies revealed that ellagic acid-loaded niosomes showed a more efficient delivery of ellagic acid through human epidermis and dermis than the ellagic acid solution, indicating that these niosomes may be a potential carrier for the dermal delivery of ellagic acid. Tretinoin cutaneous delivery is strongly affected by vesicle composition and thermodynamic activity of the drug. In particular, small, negatively charged niosomal formulations, which are saturated with tretinoin, have shown to give higher cutaneous drug retention (Manconi et al. 2006). Nasr et al. (2008) studied multilamellar liposomes and niosomes of aceclofenac, a potent analgesic, antipyretic, and anti-inflammatory agent. A comparative study was performed through evaluation of entrapment efficiency, particle size, shape, differential scanning calorimetry, in vitro drug release, and 3 months stability. Results proved that niosomes possess better stability than liposomes. Both vesicular systems showed considerable sustained anti-inflammatory activity compared to the commercial product. However, niosomes were superior to liposomes as clearly shown with both edema and inhibition rates assessed by the rat paw edema technique. Ketoprofen was encapsulated in niosomes of Span® 60 for topical application which released the drug in a slow and sustained manner (Arora and Sharma 2010). Elastic and nonelastic niosomes of gallic acid were prepared, and nonelastic niosomes showed a slight increase in the entrapment efficiency. Elastic niosomes showed, however, an increased permeation through the skin which will be beneficial for topical antiaging treatment (Manosroi et al. 2011). Manosroi et al. (2012) prepared cationic niosomes encapsulating an extract of a semipurified fraction of *Oryza sativa*. They investigated physicochemical characteristics and transfollicular penetration of niosomes through porcine skin using the follicular closing technique by Franz diffusion cells. The results of this study confirmed efficient transfollicular delivery of unsaturated fatty acids using cationic niosomes as well as the advantage of low systemic effect of a semipurified fraction of *Oryza sativa* compared to the neutral niosomes. Fluconazole-loaded niosomes of Span® 40, Span® 60, and polyoxyethylene (2) stearyl ether (known as Brij® 72) surfactants were prepared and evaluated. The prepared formulation accumulated the drug in the skin forming localized drug depots, thereby releasing the content in a sustained manner (Gupta et al. 2011). Jayraman et al. (1996) studied the topical delivery of erythromycin from various formulations including niosomes in hairless mouse in vivo. The penetration study, and confocal microscopy, revealed that nonionic vesicles could target pilosebaceous glands. A natural compound with an efficacious anti-inflammatory activity, ammonium glycyrrhizinate was loaded into bola-niosomes. These drug-loaded niosomes provided a noticeable improvement of the in vivo anti-inflammatory activity of the drug (Paolino et al. 2007).

Presence of 50 % alcohol in a marketed gel of naftifine hydrochloride, an antifungal highly lipophilic drug, has been detrimental to the skin after repeated exposure. An alcohol-free niosomal gel containing naftifine hydrochloride has been developed, and optimized to achieve maximum entrapment efficiency coupled with stability. Negatively charged niosomes have also been incorporated into a hydroxyethylcellulose gel (Barakat et al. 2009). Topical immunization with cholera toxin B is a potential

adjuvant for cutaneous immune responses when coadministered with the hepatitis B surface antigen (HBsAg)-encapsulated niosomes. Niosomes for topical delivery of vaccines using HBsAg as an antigen and cholera toxin B as an adjuvant can be effective as topical delivery of vaccines (Maheshwari et al. 2011). Mannosylated niosomes were formulated as a topical vaccine carrier system and adjuvant for the induction of both humoral and cellular immunities (Jain et al. 2005).

Aceclofenac niosomes have also been prepared for topical use after their incorporation into a carbomer gel. The niosomal gel showed improved drug penetration and therapeutic efficacy of the drug (Solankia et al. 2010). A niosomal gel containing nimesulide was compared to a plain nimesulide gel in terms of drug delivery. It was concluded that the niosomal gel showed a prolonged drug release of nimesulide, thereby enhancing the anti-inflammatory activity (Shahiwala and Misra 2002).

Estradiol-loaded niosomes obtained from nonionic *n*-alkyl polyoxyethylene ether surfactants (C_nEO_m) and cholesterol facilitated estradiol transdermal permeation (Hofland et al. 1994). Similarly, a meloxicam niosomal gel produced a greater reduction in edema in albino rats when compared to the conventional meloxicam gel due to the penetration of niosomes into the deeper layers of the skin. Meloxicam was entrapped in the niosomal gel which showed decreased side effects and increased pharmacological activity, thus proving to be a promising vehicle for transdermal delivery and an alternative to the conventional dosage form (El-Menshawe and Hussein 2013).

10.4 Proniosomes as Carriers in Percutaneous Drug Delivery

Proniosomes represent a significantly improved vesicular delivery system as physical stability problems, such as aggregation or fusion of vesicles and leaking of entrapped drugs during long-term storage are eliminated. Proniosomes are dry formulations of water-soluble carriers (vesicles) that are coated with surfactants and rehydrated to form niosomal dispersions immediately before use on brief agitation in hot aqueous media within minutes. Proniosomes are convenient to store, to transport, and to dose (single dose). Since they have similar release characteristics as conventional niosomes, they may offer improved bioavailability of some drugs with poor solubility (controlled release formulations) or reduced adverse effects of some drugs. Since proniosomes are a dry powder, further processing is possible. To provide convenient unit dosing, the proniosome powder may be processed to make beads, tablets, or capsules. The hydration of the proniosome powder is much easier than the long shaking process required to hydrate surfactant in the conventional dry film method of niosome preparation. The resulting niosomes are very similar to conventional niosomes and more uniform in size (Akhilesh et al. 2011; Hu and Rhodes 1999). They offer a versatile vesicle delivery concept with the potential for transdermal drug delivery. They form niosomes following topical application under occlusive conditions, due to hydration by water from the skin itself. There are some factors such as hydration temperature, choice of surfactant, nature of membrane, nature of drug, etc. that can affect significantly the physical properties of proniosomes.

Constituents which are selected for the preparation of proniosomes should have following characteristics: free-flow ability, nontoxicity, poor solubility in the loaded mixture solution, and good water solubility for ease of hydration. Different components, such as nonionic surfactants and membrane stabilizers, are used for the proniosome preparation, and they are shown in Fig. 10.2 (Pandey 2011).

The proniosomes are prepared by different methods:

(a) *Slurry method*: Coating material, principally maltodextrin powder, is added to the surfactant solution directly to form slurry. The solvent is dried until the powder appears to be dry and free flowing. Proniosome

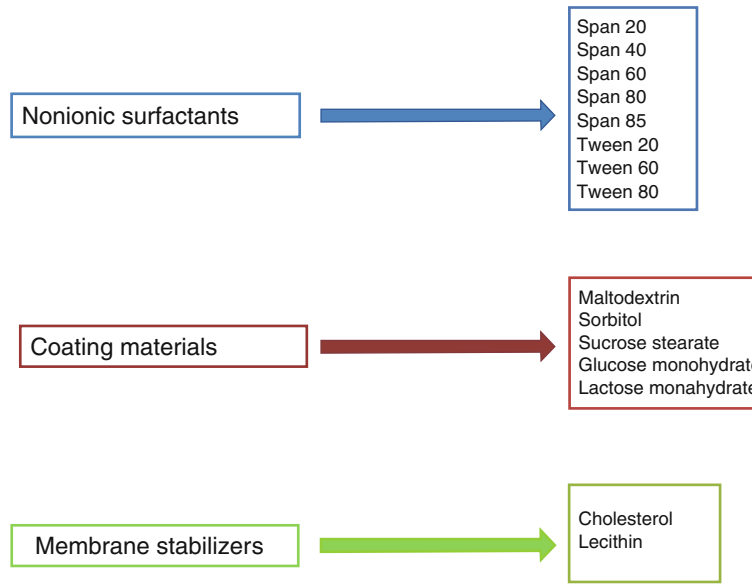

Fig. 10.2 Typical components used for preparation of proniosomes (Adapted from Pandey 2011)

powder is stored in sealed containers at 4 °C. The time required to produce proniosomes is independent on the ratio of surfactant solution to coating material and appears to be scalable (Almira et al. 2001). A proniosome formulation based on maltodextrin was recently developed and has a potential application for delivering hydrophobic or amphiphilic drugs. The better of these formulations used a hollow particle with exceptionally high surface area. The principal advantage with this formulation was that the amount of carrier required to support the surfactant could be easily adjusted, and proniosomes with very high mass ratios of surfactant to carrier could be prepared (Almira et al. 2001).

(b) *Coacervation-phase separation method*: Weighed amounts of surfactant, lipid, and drug are dissolved in hot (60–70 °C) alcohol. Then the aqueous phase (0.1 % glycerol solution) is added and warmed on a water bath till a clear solution is formed which is then converted into a proniosomal gel upon cooling (Benson 2006; Vora et al. 1998).

(c) *Slow spray-coating method*: This method involves preparation of proniosomes by spraying the surfactant in organic solvent onto coating materials, such as powder of sorbitol, and then evaporating the solvent. The surfactant coating on the carrier is very thin, and hydration of this coating allows multilamellar vesicles to form as the coating material dissolves. The resulting niosomes are very similar to those produced by conventional methods, and the size distribution is more uniform (Abd-Elbary et al. 2008; Biju et al. 2006).

In addition to dry granular type of proniosomes, it is possible to obtain the liquid crystalline proniosomes. When the surfactant molecules are kept in contact with water, there are three ways through which lipophilic chains of surfactants can be transformed into a disordered liquid state called lyotropic liquid crystalline state (neat phase). These three ways are increasing temperature at kraft point (Tc); addition of solvent, which dissolves lipids; and use of both temperature and solvent. Neat phase or lamellar phase contains bilayers arranged in overlapping sheets within intervening aqueous layers. These types of structures give typical X-ray diffraction and threadlike birefringent structures under polarized microscope. The liquid crystalline proniosomes or the proniosomal gel acts as a reservoir for dermal/transdermal delivery of drugs (Bharti et al. 2012).

Advantages of proniosomes over niosomes include (Pandey 1996; Sudhamani et al. 2010):

1. Avoiding problems of physical stability, like aggregation, fusion, leaking.
2. Avoiding hydration of encapsulated drugs which limits the shelf life of the dispersion.
3. Proniosomes are water-soluble carrier particles that are coated with surfactant and can be hydrated to form niosomal dispersions immediately before use on brief agitation with hot aqueous medium. Proniosomes (dry niosomes) can be a promising industrial product because of the facility of transportation, distribution, and storage.
4. Unacceptable solvents are avoided in proniosomal formulations. The systems may be directly formulated into transdermal patches and do not require the incorporation of vesicles into a polymeric matrix.
5. Ease of storage makes proniosomes a versatile delivery system for a wide range of active compounds.

10.5 Percutaneous Applications of Proniosomes

The importance of proniosomes can be described on the basis of different studies related to specific applications of proniosomes as a carrier system in transdermal delivery of different drugs.

Ketorolac, a potent nonsteroidal anti-inflammatory drug, is formulated as a proniosome gel using Span®, Tween®, lecithin, and cholesterol with ethanol as a solvent. Each of prepared proniosome formulations showed significantly improved drug permeation. Entrapment efficiency of drugs in prepared niosome formulations was about 99 %, and it was concluded by Alsarra et al. that proniosomes may be a promising carrier for ketorolac (Alsarra et al. 2005). Mokhtar et al. (2008) formulated a proniosomal gel of flurbiprofen using different Span® surfactants without and with cholesterol. The influence of different processing and formulation variables on flurbiprofen entrapment efficiency, such as cholesterol content, structure of nonionic surfactants, drug concentration, total lipid concentration, and the pH of the hydration medium, were studied. It was concluded that niosome formulations containing 10 % cholesterol were most stable among all the prepared formulations. Chandra and Sharma (2008) formulated piroxicam proniosomes using Span® surfactants, cholesterol, lecithin, and isopropyl alcohol. It was suggested that proniosome vesicles transfer drug from vesicles to the skin and that the penetration enhancement may be due to the effect of surfactants. Thakur et al. (2009) prepared proniosomes using different esters of sorbitan and polysorbates (Tween are registered trademarks of ICI Americas), such as Span® 20, Span® 40, Span® 60, Span® 80, Tween® 20, Tween® 40, and Tween® 80, for transdermal delivery of losartan potassium. The best in vitro skin permeation profile was obtained with proniosomes prepared using Span® 40. Proniosomes were used as carriers for transdermal delivery of lisinopril dihydrate by Shamsheer et al. (2011). The results of the this study indicated that the lisinopril dihydrate proniosomal gel containing lecithin, cholesterol, and a combination of two different Span® surfactants like Span® 20 and Span® 40 or Span® 60 and Span® 80 provided a sustained drug release over a period of 24 h for the control of hypertension. The proniosomal gel could be an effective alternative vehicle for delivering the drug by the transdermal route to avoid side effects associated with oral route. Gupta et al. (2007) investigated the potential of proniosomes as a transdermal drug delivery system for captopril which is used for the treatment of hypertension. The drug was encapsulated in various proniosomal gels composed of different ratios of sorbitan fatty acid esters, cholesterol, and lecithin. The authors concluded that these proniosomes are promising for prolonged delivery of captopril, have reasonably good stability characteristics, and can reduce the side effects associated with captopril. Fang et al. (2001b) reported about the skin permeation of estradiol from various proniosomal gel formulations across excised rat skin. Presence or absence of cholesterol in the lipid bilayers of vesicles did not reveal difference in encapsulation efficiency and permeation of the

associated estradiol. The type and content of nonionic surfactants in proniosomes are important factors affecting the efficiency of transdermal delivery of estradiol. The study suggests that inclusion of surfactants and lecithin in vesicles may play a more important role than inclusion of cholesterol on estradiol permeation. El-Laithy et al. (2011) studied a novel sustained release proniosomal system using sugar esters as nonionic surfactants. Proniosomes were converted into niosomes loaded with vinpocetine upon skin water hydration following topical application under occlusive conditions. The researchers reported that proniosomes composed of sugar esters could be considered as very promising candidates for improving the transdermal delivery of vinpocetine.

Proniosomal gel of valsartan, an ACE inhibitor, was prepared by Kakkar et al. (2011). They evaluated the vesicle size and entrapment efficiency of the drug, and performed diffusion and stability studies of the gel. Results have shown that the surfactant type and the content of cholesterol and lecithin affect the encapsulation efficiency and the drug release rate from proniosomes. In particular, the encapsulation efficiency of valsartan of proniosomes formed by Span® 60 was observed to be higher compared to that obtained with Span® 40.

Azeem et al. (2008) studied the permeation-enhancing mechanism of the proniosomal gel of frusemide (or furosemide), in which Span®, lecithin, diacetyl phosphate, and cholesterol were used as constituents. The authors studied the effect of various formulation variables on the transdermal drug flux, amount of drug deposited in the skin, and plasma level of drug. The skin permeation studies were conducted on rat skin and human skin for quantification of permeation parameters. Overall findings suggested that the proniosomal gel was able to sustain the drug level in the blood and offer a promising means for noninvasive delivery of frusemide. Varshosaz et al. (2005) developed a proniosomal gel for transdermal delivery of chlorpheniramine maleate. The system was formulated with Span® 40 and evaluated for the effect of the composition, type of surfactants used, and alcohols on the drug loading, rate of hydration, vesicle size, polydispersity index, entrapment efficiency, and drug release across cellulose nitrate dialysis membrane. It was concluded that lecithin produced more stable and larger vesicles with higher loading efficiency of the drug. The proniosomes that contained Span 40/lecithin/cholesterol prepared by ethanol showed optimum stability, loading efficiency, particle size and release kinetic suitable for transdermal delivery of chlorpheniramine maleate. Proniosomes were used as carriers for the delivery of poorly water-soluble drugs, like celecoxib. Alam et al. (2010) prepared a proniosomal gel containing celecoxib using Span® 40 and Span® 60, cholesterol, and lecithin. The hydroxypropyl methyl cellulose (HPMC) gel (4 % w/v in ethanol) was selected as a suitable base to incorporate proniosomes into a formulation. Their results indicated that proniosomes are a promising carrier for celecoxib used at low dose for transdermal delivery that can save the recipient from the harm of large doses with improved bioavailability by bypassing the hepatic first metabolism.

Conclusion

Delivery of unstable and potent active ingredients is always a major challenge for scientific researches. In this case, selection of a carrier and a suitable route of administration for better performance are crucial aspects for the delivery of drugs. For these purposes, vesicular drug delivery systems including niosomes and proniosomes have been developed, and these drug delivery systems have been demonstrated to be promising controlled drug delivery systems for percutaneous administration of drugs. Niosomes are nontoxic and nonimmunogenic drug carriers which have been widely studied for percutaneous drug delivery. Niosomes appeared superior systems over other carriers, due to their cost-effectiveness, abilities to enhance the penetration of drugs, provide a sustained pattern of drug release and nontoxic profile, and localize drug in the skin. A proniosomal formulation which is converted into niosomes represents an innovative drug delivery system. Proniosomes were also found free from aggregation, fusion, leaking, and

sedimentation of vesicles, indicating a good stability profile. These are actually a nonaqueous form of niosomes which is converted into niosomes after hydration. Proniosomes can be prepared easily by various techniques; they show enhanced stability of the formulation and simplicity in handling, they enhance drug bioavailability, and they enable controlled and prolonged drug delivery. Some studies have also shown that, for particular drugs, proniosomes show higher permeation-enhancing ability than niosomes. As niosomes were firstly exploited as a cosmetic delivery system, they still have an extensive use in that field that needs to be further explored. The topical administration of niosomes can support a large variety of relevant developments and medical applications, because their delivery-enhancing characteristics can be easily modulated by changing their composition and structure. Moreover, vesicles as dermal drug delivery systems offer many opportunities for innovative research, aimed at both increasing efficiency and reducing toxicity of drugs through simple topical application.

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11 Nanostructured Lipid Carriers (NLC): The Second Generation of Solid Lipid Nanoparticles

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11.1 Introduction: Why Do We Need Solid Lipid Nanoparticles?

There are basically two main reasons for nanocarriers in topical formulations:

1. Nanocarriers are required for *improvement of the delivery* of molecules, e.g.,
   (a) Increased penetration
   (b) Prolonged release
   (c) Solubilization (e.g., dissolution of hydrophobic molecules in lipidic nanocarrier)
   (d) Reduced side effects (e.g., irritation)
   (e) Targeting (localization in, e.g., epidermis, reduced systemic uptake)
2. Nanocarriers are used for *market differentiation*, e.g.,
   (a) Difference to competitor product
   (b) Intellectual property (IP) aspects
   (c) Marketing (e.g., "fancy" carrier in cosmetics to keep the marketing people "happy")

There are quite a number of nanocarriers for these purposes already on the market. The question arises: Do we really need a new nanocarrier? One more nanocarrier in addition to the many nanosystems already available?

First, let us define what a nanocarrier is, because there are different understandings. A nanocarrier in the broadest sense is a particle (in liquid, solid, or supercooled state) with a size of a few nm (above molecule size) to below 1000 nm (=1 μm), i.e., having a size in the nanometer range. A nanoparticle according to legal definitions by the authorities in the United States (US) and European Union (EU) (2011; US Food and Drug Administration (FDA) 2011) is a particle with a diameter below 100 nm (details cf. Chap. 18), i.e., not all nanocarriers are nanoparticles according to the law. This has important implications for labeling the products. If one does not want to have legally a nanoproduct, one needs to use nanocarriers well above 100 nm.

Based on this definition, analyzing the nanocarriers available reveals that only a few are in broader use, primarily nanoemulsions and liposomes, micelles, nanocrystals, pigments (e.g., TiO_2), liquid crystalline structures, and to some extent polymeric nanoparticles. Each of the systems has its advantages and disadvantages. The aim when developing the lipid nanoparticles was to combine key advantages of some of these nanocarriers in one delivery system, because often 2–3 key advantages were required for a product but not realizable in a single carrier system (e.g., combining advantages of cheap large-scale production (available for nanoemulsions) and chemical stabilization of active ingredient (e.g., realized by the solid matrix of polymeric nanoparticles)). After the first generation, the solid lipid nanoparticles (SLN), meanwhile the second generation was developed, the nanostructured lipid carriers (NLC) (cf. 11.2).

Of course it is not possible to generate a perfect "all rounder nanocarrier," a "jack of all trades device," or as we say in Germany an "eierlegende Wollmilchsau" ("egg-laying wool milk swine"). Of course, solid lipid nanoparticles have limitations, but their clear advantages are as follows:

1. *Cost-effective large-scale production* by high pressure homogenization (identical to the production of nanoemulsions and milk, a liter of homogenized milk can be produced for a retail price less than 0.50 € (=0.40 US$) in Germany)
2. *Composed of legally accepted excipients, e.g., listed in the FDA GRAS list* (e.g., in contrast to many polymers which are not regulatorily accepted)
3. *Low-cost excipients* for production
4. *Modulation of release* of actives due to solid state of particle matrix (identical to polymeric nanoparticles)
5. *Protection of chemically labile actives* (also due to solid state of particle matrix)
6. *High physical stability* in final formulations (no stability problems as with liposomes, e.g., in w/o emulsions)
7. *Proven feasibility to produce cosmetic products* as shown by many successful cosmetic products on the market (dermal cosmetics)
8. *Ease of incorporation into dermal formulations*

Of course, each delivery system has limitations. Examples for limitations of lipid nanoparticles are the complexity of the crystalline matrix, lipid polymorphism and its control, and restriction mainly to lipophilic actives, while hydrophilic actives can only be incorporated by solubilization in low concentrations (e.g., interferons, enzymes (Almeida et al. 1997)). For details it is referred to SLN and NLC reviews (Müller et al. 1995, 2000c, 2011b, 2007; Pardeike et al. 2009).

11.2 A "Time Travel" Back into the Solid Lipid Nanoparticle History

When dealing with a delivery system, it appears sensible to know a little bit about the history, and the persons involved, associating names with a technology. The first research work started around 1990 parallel in Germany and in Italy. In the Pharmacy Department at Kiel University/Germany, Prof. Rainer H. Müller and at that time PhD student Stefan Lucks produced nanoparticles from solid lipids (SLN) by high pressure homogenization. This resulted in a patent application on the 18th September 1991, granted in Europe on the 20th March 1996 (Lucks and Müller 1996). In parallel Prof. Maria Rosa Gasco (Fig. 11.1, left) developed also lipid nanoparticles at the Pharmacy Department at the University of Turin/Italy. She produced them via a microemulsion technique and precipitated them by pouring the hot microemulsion into cold water. Her patent application on her production method was submitted about 1 month earlier on the 5th August 1991, the US patent granted in 1993 (Gasco 1993). The same delivery system was developed in parallel in two countries, but using different production methods.

Fig. 11.1 Prof. Maria Rosa Gasco (†2013) from Turin/Italy, the inventor of SLN produced by microemulsion technology (*left*) and the SLN on the cover page of the German Pharmaceutical Journal on 3rd December 1998 (*right*) (With permission by PharmaSol Berlin)

At the beginning, the delivery system was called differently in the various first publications, e.g., lipid nanospheres (Lucks and Müller 1996), lipid nanoparticles (Gasco 2007), and even lipid microparticles, microspheres, or nanospheres (Gasco 1993, 2002; Miglietta et al. 2000). The similar particles developed by Prof. "Avi" Domb from Jerusalem/Israel were called lipospheres (Domb 1993; Masters and Domb 1998). On initiative of Rainer H. Müller around 1993, the main researchers active in this field agreed all to name these particles "solid lipid nanoparticles," abbreviated SLN – the name was born.

The intellectual property (IP) of SLN produced by high pressure homogenization (HPH) was taken over by the German company medac GmbH in Hamburg and offered on the market for licensing (Fig. 11.2, left). In the following years, SLN got more and more popular (Fig. 11.2, right), and in 1999 the technology was acquired by SkyePharma PLC (UK) as their proprietary delivery technology. The "Gasco technology" producing SLN via microemulsions was pursued, e.g., by the Italian company Vectorpharma in Trieste (later acquired by Eurand in Milan/Italy), run at that time by Dr. Fabio Carli. Vectorpharma scaled up successfully the microemulsion production method for industrial use. Prof. Gasco developed intensively the microemulsion technology further and successfully incorporated even hydrophilic actives (e.g., (Bargoni et al. 2001; Cavalli et al. 2002; Zara et al. 2002)) and used the technology also for gene delivery (del Pozo-Rodriguez et al. 2009a, b).

In 1999 the patent application for the second-generation technology, the nano(structured) lipid carriers (NLC), was filed by PharmaSol GmbH Berlin (Germany) (Müller et al. 2000a, b). Based on the experiences with SLN, the nanoparticles were improved regarding drug loading and stability of long-term drug incorporation. This resulted in the first cosmetic dermal products entering the market in 2005 (Müller et al. 2007).

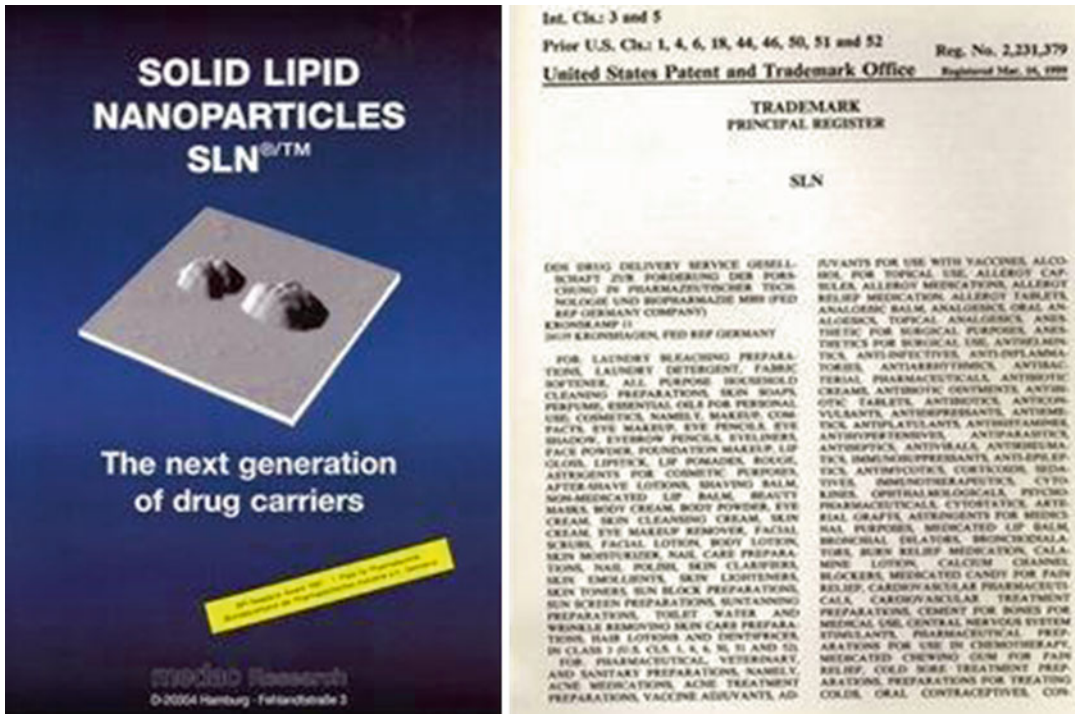

Fig. 11.2 Historical marketing flyer (around 1996) for SLN by the company medac GmbH Hamburg (Germany) (*left*) and registration of SLN as trade mark in the United States on 1999 (*right*)

11.3 Difference Between SLN and NLC/Ultrasmall NLC

11.3.1 Solid Lipid Nanoparticles (SLNs)

The SLNs are produced from solid lipids only, either glycerides or waxes, or mixtures thereof. In general only one solid lipid was used. Examples are triglycerides with different chain lengths (e.g., Dynasan series, Cremer Oleo, Germany), glyceryl dibehenate (Compritol 888 ATO, Gattefossé, France), or carnauba wax and beeswax. Characteristic is that all lipids are solid at body temperature of 37 °C, or at least at skin temperature. The drug is incorporated by melting the lipid and dissolving the drug in the melt, and then the nanoparticles are produced, typically by hot high pressure homogenization in the melted state, the so-called hot homogenization method. The lipid melt droplets containing the drug recrystallize during cooling, yielding typically a mixture of α, β′, and β lipid modifications of the lipid. During storage, the lipid molecules tend to increase their order and transform from α/β′ to β modification. This can lead to the expulsion of the drug from the now more perfect lipid particle matrix. This expulsion effect occurs directly during or immediately after the cooling process when high purity lipids are used such as trimyristin. The particles solidify, the lipid crystallizes highly ordered, the drug is expelled, and drug crystals are formed in the water phase of the SLN suspension (Fig. 11.3) (Bunjes et al. 1996).

11.3.2 Nano(Structured) Lipid Carriers (NLC)

Less perfect crystals can be formed when the lipid molecules are different in molecular size and conformational shape. Based on this consideration, lipid nanoparticles were made from lipid blends by blending structurally very different lipid molecules, i.e., solid lipids with longer chain fatty acids and oils with shorter chain fatty acids (e.g., MCT oils – medium-chain triglycerides). To differentiate them from SLN, they were termed "nanostructured lipid carriers" (NLC). Of course, the melting point (MP) of the blends still needs to be above body/skin temperature. To have a high content of oil, it is preferred to blend high melting lipids such as carnauba wax (close to 90 °C) with oils, which allows to have up to two third of oil in the blend. The melting point can be checked by differential scanning calorimetry (DSC) of the bulk mixture. However, when selecting the composition of the blends, it needs to be kept in mind that there is a melting point (MP) depression when the material is in nanosize – compared to bulk material (Thomson equation). Nanomaterial melts at lower temperature than bulk material. Thus, when screening the bulk lipid mixtures, they should have an MP about 5 °C higher than skin temperature to make sure that in nanosize the MP is still above 32 °C! The less perfect crystalline matrix with a high number of imperfections provides more space to accommodate the drug. The drug load can be increased, e.g., as shown for retinol from 1 % in SLN to 5 % in NLC (Jenning and Gohla 2001). In addition, drug expulsion during storage is avoided or reduced due to this higher drug incorporation capacity.

11.3.3 Ultrasmall NLC

Typical sizes of SLN and NLC are around 200–400 nm. This is a size easily accessible by high pressure homogenization (HPH). Sizes in the

Fig. 11.3 SLN can form a highly ordered crystalline structure (like bricks in a wall) leading to drug expulsion (*left*), while the less ordered lipid matrix of NLC can better accommodate drugs

upper nm range (e.g., >700 nm) are less stable due to, e.g., flotation and creaming on long term. Producing sizes below 200 nm requires higher surfactant concentrations, which is often not desired. In addition, lipid nanoparticles <<100 nm have problems as they tend to recrystallize. Options to initiate crystallization are supercooling below room temperature (e.g., refrigeration or freezing and subsequent thawing). However, for some applications, ultrasmall NLCs (<<100 nm) are of high interest, i.e., for the penetration into the skin (Schwarz et al. 2012). Recently they were developed using the method of matching the required HLB of the lipid phase (Baiseng 2013, in preparation). In addition they were found to enhance the activity of actives, e.g., the antioxidant activity of Q10 in ultrasmall NLC (usNLC) was about 15-fold when compared to classical NLC (Keck et al. 2014).

11.4 Production on Lab Scale

11.4.1 Production Methods

The production methods for SLN and NLC are identical, just the starting material is different. The solid lipid of SLN is replaced by a lipid blend of solid lipid and oil. The currently most used methods are HPH and the microemulsion technique.

In HPH the particles are produced similar to the homogenization of milk. The drug is dissolved or dispersed in the molten lipid, and then a pre-emulsion is formed by dispersing the drug-loaded hot lipid melt in a surfactant solution of equivalent temperature by high-speed stirring. The obtained pre-emulsion is passed through a high pressure homogenizer. Typical pressures are 500–800 bar; 3 to 1 passages (cycles) are normally sufficient to obtain nanoparticles between 200 and 300 nm. The obtained hot nanoemulsion is then cooled in a controlled way, and then lipid/lipid mixture recrystallizes to form SLN or NLC.

In the microemulsion process, a hot microemulsion is formed composed of melted lipid with surfactant/surfactant-cosurfactant and water, which is then poured into cold water. The microemulsion breaks by this dilution with water-forming nanoparticles. At the same time the lipid recrystallizes in the cold water. Advantage of this process is that no expensive equipment (homogenizer) is necessary. Therefore, this process is very popular in less well-equipped labs. Disadvantage of the process – especially for industrial production – is the dilution of the nanoparticle suspension by dilution in the water, resulting in concentrations of a few percent or less. In contrast, HPH can produce up to 50 % concentrated suspensions, favorable to be admixed as concentrates to creams.

Industrially most feasible is high pressure homogenization (HPH), but in academic research often other methods are used which do not require the expensive equipment of a high pressure homogenizer (approx. 20,000–40,000 US$). A highly suitable lab homogenizer is the hydraulically driven Micron LAB 40 (APV Deutschland GmbH, Germany and APV Denmark) (Fig. 11.4). The Micron LAB 40 has the advantage of a batch size of about 20–40 ml. This is sufficient to generate the necessary sample quantity for, e.g., stability studies on storage (e.g., 3 samples of 10 ml at 3 temperatures), and not wasting too much material because it can be run with a low volume. Other homogenizers require often more than 100 ml, which can be costly in formulation screening when expensive materials are used. However, in principle each homogenizer suitable for the production of nanoemulsions can be used.

11.4.2 Lipid Screening

Important is the lipid blend screening. It should be ensured that the mixture of solid and liquid lipid is uniform (one phase) in the molten state but – most important – also in the solid state. For certain NLC structures, lipid blends can be used which are miscible in the liquid state, but on purpose are not miscible in the solid state (e.g., core-shell particles). The screening is performed by using different ratios of solid and liquid lipid, by melting and mixing them, and finally macroscopic evaluation of the lipids in the molten state is performed. Then the blend is casted in a thin

Fig. 11.4 Micron LAB 40 homogenizer for lab scale production of lipid nanoparticles: Homogenization tower (*left*) with homogenization chamber in the middle and self-built temperature control jacket placed below, through-flow of hot water (typically 70–95 °C). In the back control panel and digital pressure display

film onto a microscope slide and inspected after solidification (macroscopically and microscopically). If the film is turbid, or large crystals are seen, then the mixture has been separated in a blended phase and a second lipid phase (oil or solid lipid crystals). When this separation occurs in the bulk phase on the slide, it also occurs in the NLC particles. This effect in the particle matrix can be exploited for the generation of core-shell lipid nanoparticles. In addition to the "slide method," the blends can be investigated by DSC, but this simple microscopic method using a slide has proved most effective, fast, and often superior to DSC. Alternatively the melt can be generated in an injection vial. The vial should be horizontally rotated during cooling of the melt, to cast a thin lipid film on the vial wall. In this film, crystals forming due to phase separation can easily be macroscopically detected.

The same procedure is performed to check the solubility of drugs or cosmetic actives in the melt and in the solidified lipid. This is important to produce in a controlled way NLC with solid solution matrix, or with drug-enriched core or vice versa (cf. Sect. 11.9.3). If the film is homogenous without detectable crystals, the lipid film represents a solid solution (drug molecularly dispersed in lipids). When drug crystals appear in the casted film, the solubility is exceeded. That means during cooling of the hot oil droplet and crystallization process, the drug precipitates first forming a nanoparticle with drug-enriched core. Table 11.1 shows compositions of SLN and NLC for dermal use.

Table 11.1 Examples of SLN and NLC formulation (Üner et al. 2005b)

Materials	Function	SLN formulation	NLC formulation (%)
Witepsol® E85	Solid lipid	10 %	6.7
Miglyol® 812	Liquid lipid	–	3.3
TegoCare® 450	Surfactant	1.5 %	1.5
Water	Dispersant	88.5 %	88.5

11.5 Production on Large Scale: The Prerequisite for Industrial Products

Large-scale production is essential for a commercial product. The industrial production lines need to be qualified and validated (according to authority requirements) and ideally should be cost-effective. High pressure homogenization is an ideal production method because it fulfills all these criteria. In addition, HPH production lines

are already used in cosmetic and pharmaceutical industry, e.g., for the production of pharmaceutical parenteral nanoemulsions such as Intralipid (Baxter, USA) and Lipofundin (B. Braun Melsungen, Germany).

The first commercial NLC concentrates were produced with a production line composed of a Gaulin 5.5 (APV Gaulin, Germany) in combination with 60 kg feeding and product containers (Fig. 11.5). The containers possess electropolished surfaces and are double walled and temperature controlled. The molten lipid mixture and surfactant are added to the feeding container, and then sterile water from the hot sterile water supply system is added. A coarse pre-emulsion is generated by high-speed stirring (e.g., 10–20,000 rpm) with a built-in propeller stirrer. The lipid mixture is dispersed in the aqueous phase yielding a w/o emulsion. The premix is then typically passed only once through the homogenizer at a pressure between 600 and 800 bar (approx. 8700–11,600 psi). The obtained hot nanoemulsion is collected in the product container and cooled in a controlled way. If required, a heat exchanger can be placed between the homogenizer and the product container.

Figure 11.6 shows a more sophisticated design for NLC production on large scale, which also allows processing of temperature-sensitive molecules, e.g., vitamin A in cosmetics. To avoid too long heat exposure in the hot lipid blend, the vitamin A is dissolved in cold oil and admixed to the melted hot solid lipid in a static blender, before being mixed in a second static blender with the hot surfactant solution. Then the formed coarse emulsion is entering the homogenizer. The product is

Fig. 11.5 Large-scale production line for lipid nanoparticles – from *left* to *right*: feeding container, APV Gaulin 5.5 homogenizer (manufacturer, country) and control unit (both in the back), and product container at the *right*. Product containers are made of pharma steel and can be sterilized by streaming steam and run under protective nitrogen gassing. The homogenization chamber of the homogenizer can also be sterilized by steam. The control unit records continuously production parameters

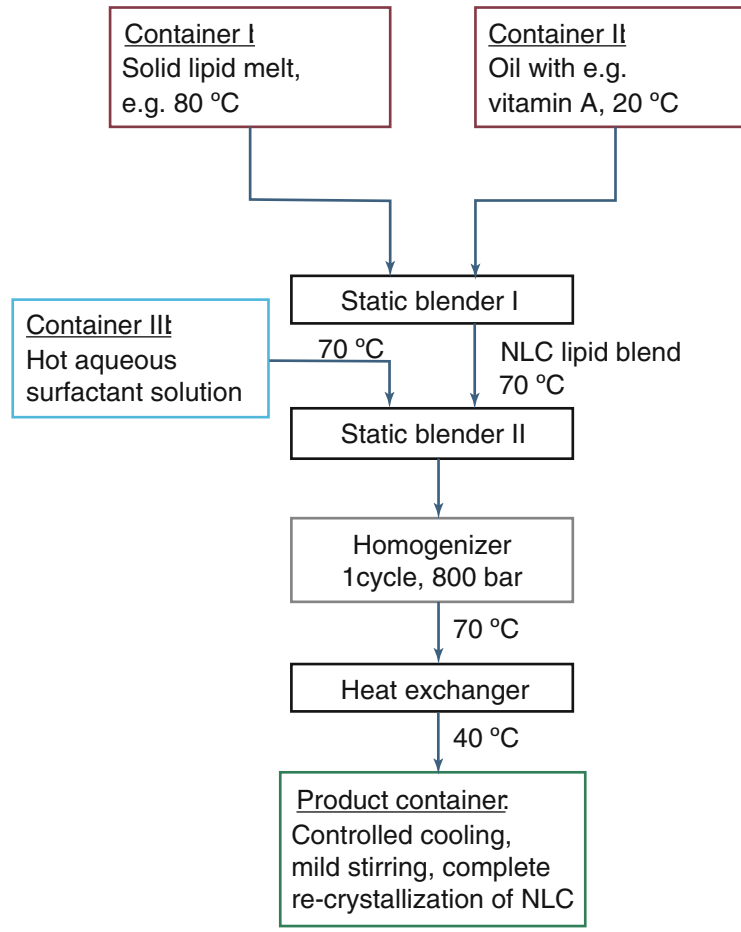

Fig. 11.6 Large-scale production line for processing of temperature-sensitive drugs/cosmetic actives (explanation cf. text)

partially cooled by a heat exchanger and recrystallizes completely in the product container under controlled cooling and mild stirring.

11.6 How Do SLN/NLC Work? Mechanisms of Action

11.6.1 The "VW Beetle 1938 Principle"

What is the *VW Beetle principle*? The VW Beetle (German: "Volkswagen Käfer") started to be built in Germany in 1938 and continued to be built until 2003 (Fig. 11.7, left). This car was characterized by its simple but very effective and reliable construction and design. Classical example is the air cooling of the engine, which proved much superior to liquid-cooled engines. The car was running without problems in the cold Russian winter of Moscow and in the hot deserts of North Africa. In addition, in case of a breakdown, the driver was able to do some repairs (not possible nowadays with the sophisticated cars).

The same applies to the lipid nanoparticles, both SLN and NLC. They are relatively easy-to-make particles, creating an increased drug penetration in a simple way, but highly effective using physical principles and chemical principles. These principles on the skin are as follows:

1. Physical *adhesion to the skin* due to their small size (increased surface interaction, e.g., van der Waals forces, hydrophobic interactions, electrostatic interaction in the case of positive particles)

Fig. 11.7 The simple but very robust and reliable old beetle invented on 1938 (*left*, model 1974) representing the basic lipid nanoparticle SLN technology and the "New Beetle" (*right*, model 1999) with a more sophisticated technology similar to NLC, but also similar to more sophisticated designed SLN (e.g., supersaturation effects by SLN)

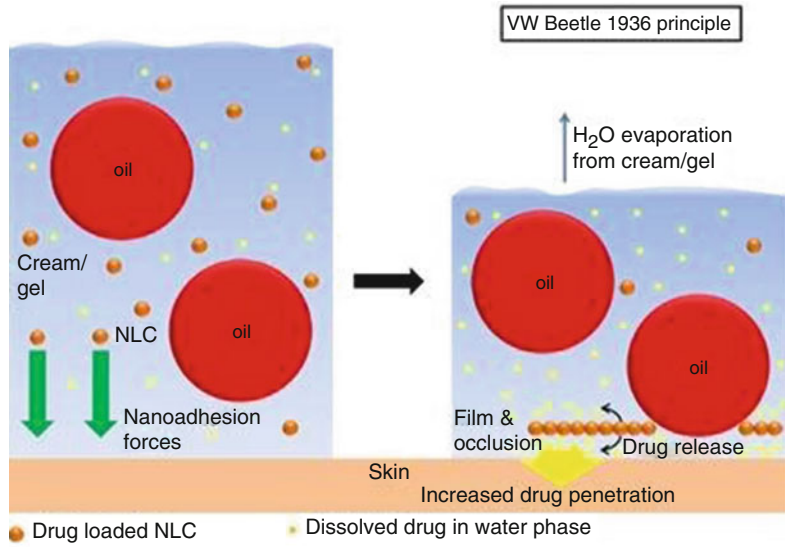

Fig. 11.8 Mechanism of lipid nanoparticles (SLN, *NLC*) adhering to skin (*left*), forming a film which is occlusive and promotes drug penetration (*right*), especially of drugs released from the nanoparticles adhered to the skin

2. Physical *film formation creating occlusion*
3. Physical *occlusion promoting drug penetration*
4. *Chemical interaction of lipids from particles with skin lipids*

The film formation is very pronounced when choosing a sufficient amount of nanoparticles in the formulation, which is able to sufficiently cover the skin surface (typically 2–4 % of pure nanoparticles in a cream). It creates a film similar to a cling film, which keeps enwrapped fruits fresh (and the skin moist). NLCs were therefore also called the "invisible dermal patch" (Sinambela et al. 2011). This occlusion improves penetration due to the well-described mechanisms (Fig. 11.8).

In addition, the lipids of the particles can optionally mix with the skin lipids, thus also affecting drug distribution between the lipid structures of the skin and affecting penetration depth. For the drug prednicarbate, localizations in different layers of the epidermis/dermis were described as a function of the lipid used for the SLN production (Maia et al. 2000, 2002). In contrast to the aforementioned physical principles 1–3 involved in the interaction of SLN and NLC with the skin, the particles' lipid – skin lipid

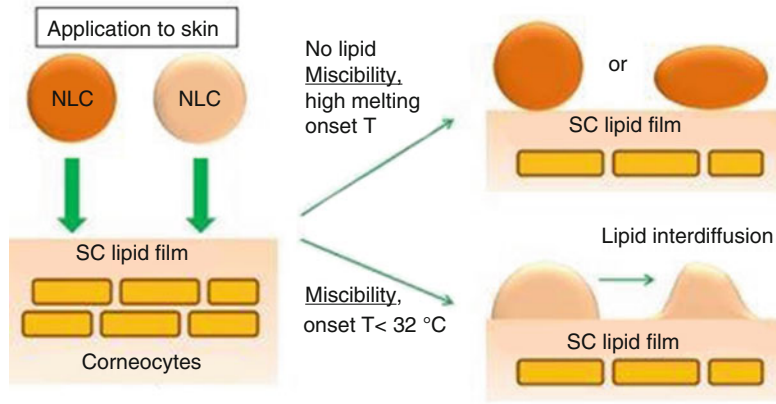

Fig. 11.9 Proposed mechanism for interaction of lipid nanoparticles with lipids of the film on the stratum corneum (*SC*): no interaction for particles with no lipid miscibility/high melting temperature (*upper right*), interdiffusion of lipids in case of miscibility, particle integration into skin lipid film by fusion, and "interdiffusion" (*lower right*)

interaction – is little understood by now and the ideal lipid matrix composition has to be found empirically. Figure 11.9 proposes a mechanism of this interaction between lipids. Lipid nanoparticles remain primarily on the skin without interaction with the skin lipids if their matrix lipids are not miscible with stratum corneum (SC) lipids, or if the particle matrix possesses a very high melting point (solid state reduces interdiffusion of lipids). The particles might only be slightly flattened by the application pressure. The interaction between lipids occurs in case of good miscibility, and especially when the onset of the melting peak of the particles' lipids is below the skin temperature. This leads to partial softening of the particle matrix promoting interdiffusion of lipids between particles and SC lipids, potentially integrating the lipid nanoparticle in the lipid film on the SC. The "*VW Beetle 1938 principle*" applies to both SLN and NLC.

It should clearly be stated that lipid nanoparticles >100 nm are not penetrating as intact particles into the skin. Applied to the skin, they stay on the surface (Schäfer-Korting et al. 2007) and can potentially be deformed on the surface by the pressure during application which promotes film formation, especially when the melting point is close to skin temperature (e.g., onset of melting peak below 32 °C). Capillary forces in between closely packed nanoparticles can also contribute to the film fusion (similar to tablet coating with polymer nanoparticle dispersions).

11.6.2 The "New Beetle Principle"

In 1997 the New Beetle was introduced to the market by the German company Volkswagen (VW), build until 2010, the retro design resembling the old Beetle (Fig. 11.7, right). The major difference was that the car technology was much more sophisticated. In contrast to the old beetle, the driver did not need to have a tool box anymore in his car, as he was not able to repair anything. However, the new technology provided much more options. The same can be achieved when designing the lipid nanoparticles in a more sophisticated way. Primarily this is possible by using SLN with a complex composition of solid lipids, or alternatively using NLC, i.e., mixing a solid lipid with a liquid lipid. The more complex mixture of lipids gives more flexibility in designing the particle matrices. Basically by selecting appropriate chemical composition of the particle matrix (1), setting trigger mechanisms (e.g., temperature increase) (2) and adjusting formulation composition (e.g., concentration of lipid and surfactant) and production parameters (3), one can:

1. *Control the polymorphic transitions of high energy to low-energy modifications (β)*
2. *Create a supersaturated solution of drug in the outer aqueous phase and optionally*
3. *Produce smaller lipid particles (both SLN and NLC)*

By generating very complex mixtures of solid lipids or mixtures of solid and liquid lipids, the

crystallization/transformation to β modification is hindered by the structurally very different molecules (Sinambella, P. 2013). This avoids drug expulsion and allows higher loadings. In addition, a trigger can be used to initiate the transform, which leads to the expulsion of the drug from the particle matrix (trigger-controlled release) and formation of supersaturated solutions of drugs. Cyclosporin A (CyA)-SLN were produced using chemical name (Imwitor 900, manufacturer, country) and highly loaded with CyA (20 %) (Radtke 2003). The CyA-loaded SLN kept the drug enclosed at room temperature, while the temperature increase to 32 °C (skin temperature) initiated the transform to triclinic β modification and expulsion of the drug to the water phase of the formulation. The water phase was already saturated with CyA, and CyA expulsion from the particle matrix led to supersaturation in the water phase (Fig. 11.10). The system tends thermodynamically to reduce the supersaturation, which is only possible by diffusion of the drug out of the water phase, i.e., drug penetration into the "acceptor phase," i.e., into the skin (= microemulsion principle of supersaturation (Müller 1998)).

The size of lipid nanoparticles decreases with increasing surfactant concentration, with decreasing lipid concentration in the suspension, and with decreasing viscosity of the lipid melt during homogenization. With the NLC technology, ultrasmall lipid nanoparticles can be obtained, e.g., NLC composed of 80 % dicaprylyl ether (Cetiol OE, BASF, Germany) and cetyl palmitate (20 %), stabilized by a surfactant mixture of Tween 80 and Span (Baiseng 2013, in preparation). The ultrasmall NLCs are of interest for enhanced penetration as shown for dermally applied 40 nm dendrimeric nanoparticles loaded with Nile red as model compound, showing enhanced penetration (Küchler et al. 2009).

11.7 Excipients: Regulatory Aspects

For many nanocarriers described in the academic literature, especially polymeric nanoparticles, the excipients used are not regulatorily accepted (e.g., FDA). They might give nice results in research, but cannot be used for products on the market, e.g., many experimental newly synthesized polymers are not allowed to be used in humans. Companies are reluctant to perform toxicity studies of a new compound; each company is waiting for the other, or waiting that an excipient producer is performing these studies. Thus, in general one tries to create formulations only by

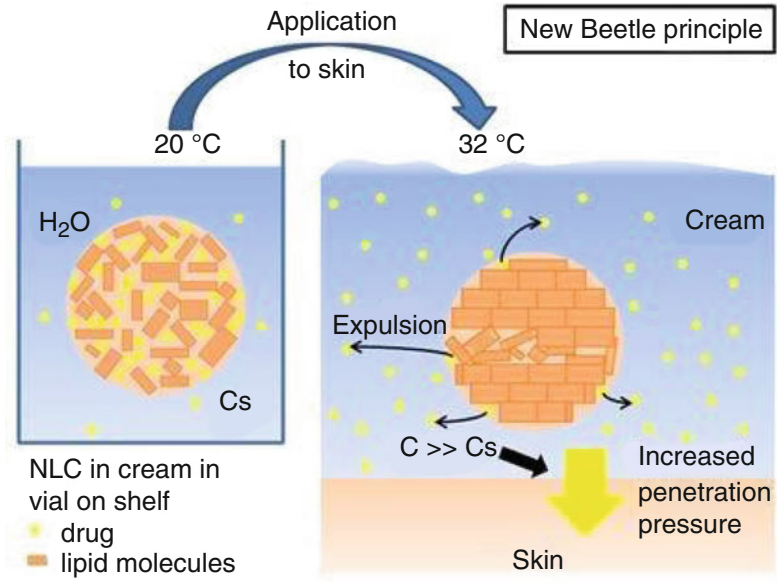

Fig. 11.10 Creation of drug supersaturation in water phase in dermally applied cyclosporine-loaded SLN: In the vial on the shelf, the particle matrix is less ordered with the drug incorporated in imperfections (*left*), and the water phase is drug saturated (c_S). Increase in skin temperature triggers the transform to a more ordered lipid particle matrix, resulting in drug expulsion into the water phase and drug supersaturation of this phase ($c >> c_S$)

using well-known and accepted excipients, which hinders the introduction of new innovative delivery systems.

In contrast to this, a "bouquet" of excipients is available to produce lipid nanoparticles. All solid lipids, liquid lipids, and surfactants/stabilizers used in cosmetic and pharmaceutical industry for dermal formulations are available! Increasing interest gained lipids and oils from plant origin (= natural, "green products," especially popular in Europe), especially when they are containing ω-3 and ω-6 unsaturated fatty acids (= providing a marketing story). "Fancy" oils are argan oil, black current seed oil (in NLC produced by CLR GmbH Germany), sacha inchi oil (from fruits of the Plukenetia volubili), and kukui oil (from *Aleurites moluccana)* from the Hawaiian Islands (e.g., in NLC of the cosmetic product nanorepair, Dr. Rimpler GmbH, www.rimpler.de).

11.8 Nanocarriers: Nanotoxicity Aspects

About 10 years ago, there was the equation "Nano = Good," and the sentence "Nano is good 4 U." Meanwhile the public perception changed; one looks more and more critical toward potential undesired effects or potential toxicity of nanomaterials. For a rough classification of the tolerability/potential toxicity of nanomaterials, the "Nanotoxicological Classification System" (NCS) was proposed (Keck and Müller 2013; Müller et al. 2011a).

In the chapter of this book about "nanocrystals," the NCS is briefly presented. It classifies nanomaterials according to a traffic light system, green, yellow, and red. Green (= class I) are nanoparticles with a size >100 nm to 1000 nm (= very limited access to cells) and are biodegradable (i.e., they degrade eventually). These nanoparticles are with potential excellent tolerability! Red (class 4) with potential high risk are particles below 100 nm (good access to many cells of the body), being non-biodegradable (i.e., they stay forever, e.g., fullerenes). The lipid nanoparticles are biodegradable, and with a particle size typically >>100 nm, they belong to the well-tolerated class I, or with a particle size <100 nm, they belong at least to class II of the NCS.

In addition, it should be pointed out that man is living with lipid nanoparticles since its existence! Each eaten fat particle will be surface degraded in the gastrointestinal tract by lipases and shrinked from the μm size to the nanometer size before its complete digestion. Thus, the lipid nanoparticles are also an ideal oral delivery system.

11.9 Performance of NLC on the Skin: The Special Features

11.9.1 Occlusion

The SC is the outermost layer of the skin consisting of protein-enriched corneocytes, compactly embedded in a continuous lipid layer (well known as the "brick mortar" model). This combination provides a barrier for water-soluble and lipid-soluble materials (Rogiers 2001; Schäfer-Korting et al. 2007). Lipid nanoparticles occlude the skin by forming a continuous thin film, when the water from the formulation evaporates, due to their adhesive properties (Müller et al. 2007). This occlusion reduces water evaporation from the skin and increases the hydration of the SC which normally has only 10–15 % water content. Consequently, the corneocytes become less compact and the inter-corneocyte gaps become wider. These changes increase the permeability of the SC and can promote drug penetration (Schäfer-Korting et al. 2007).

Although both SLN and NLC have occlusion properties, they can differ in the extent of occlusion which they provide. An in vitro occlusion study using the "de Vringer occlusion test" by Souto et al. showed that the investigated NLC had lower occlusion property than SLN at similar lipid concentrations, possibly due to less crystallinity of the matrix of NLC (Souto et al. 2004). Teeranachaideekul et al. studied the influence of the oil content in NLC on the occlusion effect on excised human skin and the resulting penetration

of the model compound Nile red (Fig. 11.11). NLC with 5–10 % oil content formed a thin film, while the NLC with 30–50 % oil formed creamy structures on the skin. With nanoemulsions, no film or creamy structure was observed on the skin (Teeranachaideekul et al. 2008). Occlusion was highest with lipid nanoparticles possessing highest crystallinity, i.e., NLC 5 and 10 % oil content, i.e., lowest oil content!

Despite differences observed in in vitro and ex vivo studies of occlusion, the in vivo study in human subjects showed that regular application of NLC increases the skin hydration, attributed to the NLC occlusion effect (Pardeike et al. 2010). Üner et al. compared the skin hydration after application of SLN and NLC on human skin. The hydrogel formulations containing SLN or NLC increased the skin hydration more than the hydrogel formulation without lipid nanoparticles, but the study did not show any significant difference in the skin hydration increase provided by SLN and NLC (Üner et al. 2005a). Other studies showed that both SLN and NLC can provide long contact time of lipid nanoparticles with the skin by showing 24-h efficacy of nitrendipine and flurbiprofen on rat after topical application (Bhaskar et al. 2009a, b). Depending on the concentration of lipid nanoparticles in the formulation, the contact time of lipid nanoparticles on human skin can reach up to 24 h (= maximum observation period) (Sinambela et al. 2011).

11.9.2 Penetration Enhancement of Actives

As outlined above, penetration enhancement of actives is strongly related to the occlusion, which was confirmed by the study of Teeranachaideekul et al. (2008). The NLC with lowest oil content (5 %, Fig. 11.11a) exhibited the best penetration of Nile red into the skin. These NLCs possessed highest crystallinity, being close in their matrix structure to SLN. All NLCs were clearly superior to the nanoemulsions which provided a low penetration of the dye.

A study performed by Puglia et al. showed that NLC loaded with ketoprofen and naproxen provided higher drug amounts in the SC compared to the drug solution after 6-h occlusive application on the skin. It was shown by tape stripping that NLCs increase the drug penetration and prolong the drug release acting as a reservoir (Puglia et al. 2008).

11.9.3 Modulation of Release of Active by Nanostructuring of Particle Matrix

Which factors are affecting the release profile from lipid nanoparticles? Actually it is a multifactorial event, where many factors interplay. In the literature some general tendencies can be derived. In general it is described that NLCs show a faster release than SLN because the lipid matrix has a lower crystallinity (Bhaskar et al. 2009a). However, both SLN and NLC showed sustained release compared to other nanosystems for skin delivery such as nanoemulsions (Mitri et al. 2011). The release profile is also influenced by the distribution of the incorporated active in the lipid particle matrix, the type of the dermal product (emulsion, gel), and the skin secretions (Schäfer-Korting et al. 2007).

The possible distribution of the active in the lipid nanoparticles is schematically shown in Fig. 11.12. The particle matrix can be a solid solution of the drug (Fig. 11.12a); drug can be enriched in the outer shell leading to fast release ("burst" effect) or in the core (Fig. 11.12b, c). Localization primarily in the core prolongs release, e.g., as shown for prednisolone in vitro (zur Mühlen et al. 1998). Surface localization/adsorption is also possible, which favors fast release (in the case when binding to the particle surface is not too strong). The molecules can be evenly distributed (Fig. 11.12d) or enriched

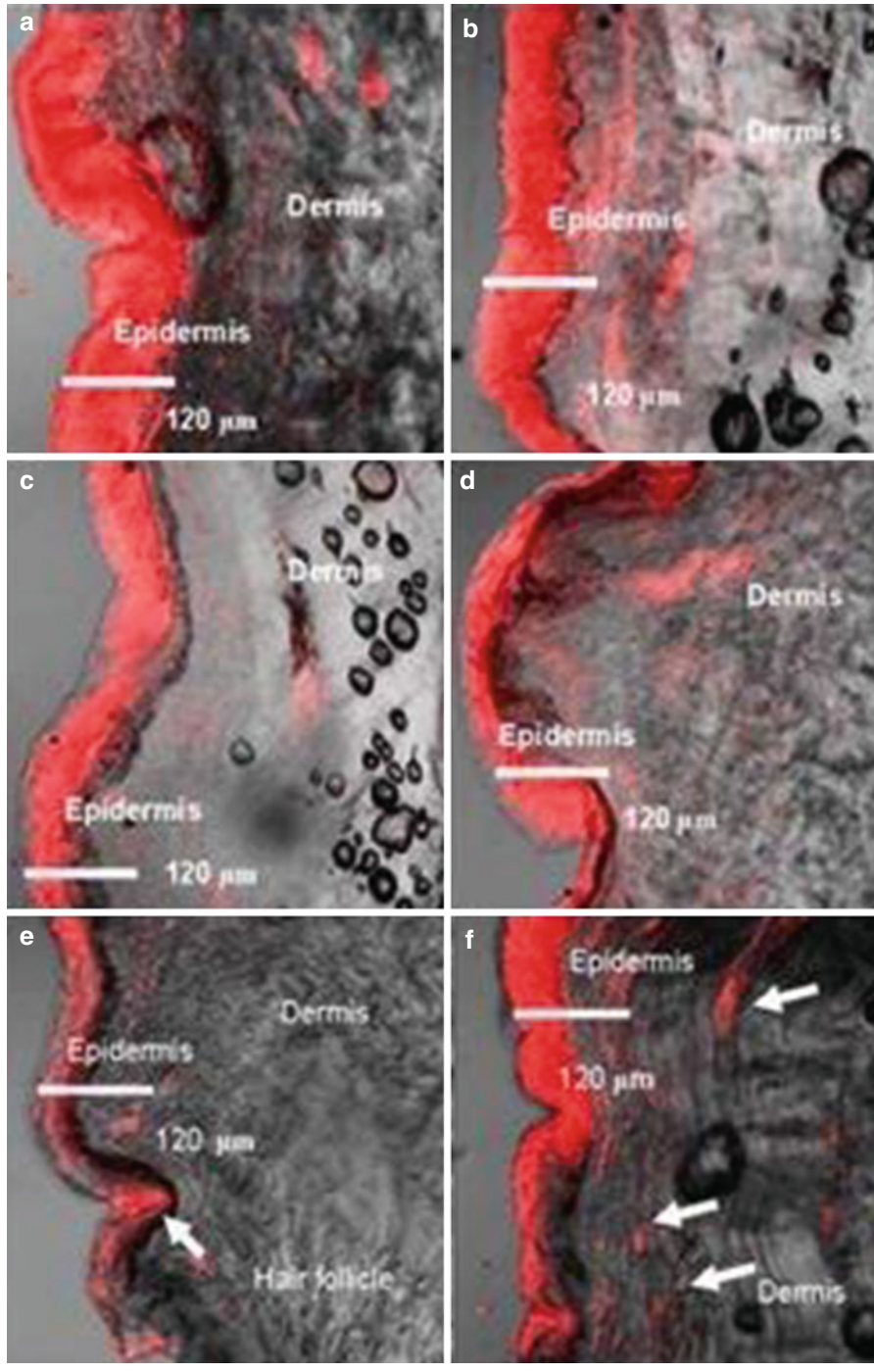

Fig. 11.11 Penetration and distribution of Nile red within human skin after the application of nanoemulsion and NLC for 3 h (**a**–**e**): (**a**) NLC-5 % oil, (**b**) NLC-10 % oil, (**c**) NLC-30 % oil, (**d**), NLC-50 % oil, (**e**) nanoemulsion and (**f**) Nile red-loaded NLC-30 % oil after 9 h (With permission after (Teeranachaideekul et al. 2008))

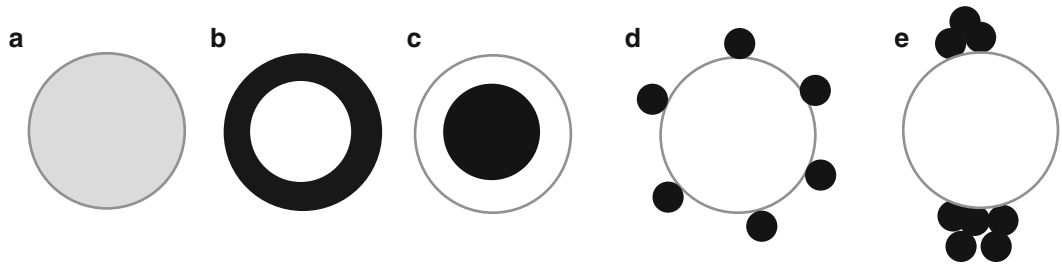

Fig. 11.12 Distribution of loaded active within the NLC lipid matrix: (**a**) monolithic solution, (**b**) enrichment of active in the lipid shell, (**c**) enrichment in the lipid core, (**d**) adsorbed monolayer of active on the lipid surface, (**e**) agglomeration of active on the lipid surface, formation of drug patches (With permission after (Schäfer-Korting et al. 2007))

clusterwise in certain areas (drug patches, Fig. 11.12e). Adsorption to the surface can also be used to load lipid nanoparticles with hydrophilic drugs, as shown with the model compound serum albumin (Schubert and Müller-Goymann 2005).

Often forgotten: Drug release can also be promoted by lipid degradation by enzymes in the skin or electrolyte change in the SC (Schäfer-Korting et al. 2007). The electrolytes might cause changes in the lipid structure of the particles, promoting transform to more ordered polymorphic form and drug expulsion.

11.9.4 Supersaturation Effects

After the lipid nanoparticles are applied on the skin, the water from the formulation starts to evaporate. This evaporation and/or the increase in temperature can sometimes promote the lipid transition to a more regular and more dense structure (polymorphic transition from β′ to β), as already outlined above. As a consequence, the loaded drug will be expelled from the particle matrix and released to the water phase on the skin (Müller et al. 2002a, b; Schäfer-Korting et al. 2007).

To exploit this effect, the water phase needs to be saturated with the drug. The supersaturation leads to an increased concentration gradient, identical to microemulsions and nanocrystals (cf. Chap. 18). The solution tries to reach its thermodynamic equilibrium solubility, which is possible by precipitation of crystals of the drug or penetration of the drug into the skin. The latter typically happens, as described for microemulsions (Müller 1998).

11.9.5 Proof of Dermal Efficiency By In Vivo Studies

Quite a number of in vivo studies have shown the ability of NLC to increase the active penetration either into the skin (dermal delivery) or through the skin (transdermal delivery) as shown in Table 11.2. The studies show potential applications of NLC to improve drug penetration and efficacy in the targeted area.

11.10 New Analytical Insights into the Structure of Lipid Nanoparticles (SLN, NLC)

As presented in the Sect. 11.9.3, there are different models of nanostructure for lipid nanoparticles. In general it can be summarized that despite 20 years of lipid nanoparticle research, only a limited number of structural investigations have been published. The advantage of microscopic techniques compared to other methods in this respect is that the nanoparticle size and shape is directly determined. However, the size of NLC is around 200 nm (see above), and resolution in microscopy is diffraction limited, i.e., whether a particle can be detected is determined by the wavelength of the light used to create the image as expressed

Table 11.2 In vivo studies for NLC formulation

Loaded active (reference)	Target delivery	Study results
Coenzyme Q10 (Pardeike and Müller 2007)	Dermal delivery	Tape stripping study in human showed that NLC increased the skin penetration of coenzyme Q10 compared to an emulsion and a liquid paraffin formulation
Ketoprofen and naproxen (Puglia et al. 2008)	Dermal delivery	Tape stripping study in human showed that NLC reduced drug penetration into the deeper layer of the skin and increased the accumulation of active in the stratum corneum; thus, it prolonged anti-inflammatory effect in the skin compared to the drug solution
Tacrolimus (Pople and Singh 2006)	Dermal delivery	The tape stripping study in guinea pig showed that NLC increased the skin penetration The study in rat showed that NLC provided negligible systemic penetration of tacrolimus The study in rabbit showed that the NLC formulation was less irritant than the marketed ointment formulation
Flurbiprofen (Bhaskar et al. 2009a)	Transdermal delivery	The study in rat showed that NLC gave higher bioavailability and higher anti-inflammatory effect than the dermal application of SLN formulation and oral delivery of drug compound
Nitrendipine (Bhaskar et al. 2009b)	Transdermal delivery	The study in rat showed that NLC increased the bioavailability of nitrendipine compared to the oral delivery of drug compound The NLC formulation gradually decreased hypertension and maintained the blood pressure compared to the oral formulation which drastically decreased the blood pressure The study in rat showed that the NLC formulation is non-irritant
Valdecoxib (Joshi and Patravale 2006)	Dermal delivery	The study in rat showed that the NLC formulation gave no irritation compared to the marketed gel formulation The study in rat showed that the NLC formulation had faster onset and prolonged effect up to 24 h compared to marketed gel formulation

in Abbe's criterion for microscopy. A conventional light microscope is limited to resolutions of ~250 nm. Electron microscopic investigations allow for the visualization of NLC ultrastructures, but are limited to nonphysiological environmental conditions since the samples are investigated under vacuum.

Recent new optical technologies can provide deeper insight into the inner lipid nanoparticle structure, based on techniques that circumvent Abbe's criterion such as stimulated emission depletion (STED) or single-molecule fluorescence localization microscopy (Betzig et al. 2006; Hess 2007; Rust et al. 2006; Hess et al. 2006). These are the so-called super-resolution microscopic techniques allowing one to investigate the sample under more physiological conditions. A combination of single particle tracking (SPT) and super-resolution fluorescence microscopy thus facilitates the determination of the shape of the inner nanostructures of NLC, the drug distribution within the NLC, and the outer shape of NLC (Keck et al. 2013).

As already mentioned above, there might be a phase separation between solid lipid saturated with oil and an oil phase saturated with dissolved solid lipid during the cooling process after the production. Such oil compartments were postulated to be distributed as nanocompartments within the particle matrix (Müller et al. 2002a, b; Jenning 1999). The solubility of the drug is higher in the oil; thus, the oil phase will predominantly contain the drug. Phase separation leading to the formation of a liquid oil core surrounded by a solid lipid shell was recently reported from a structural investigation (Keck et al. 2013). In this study, a fluorescent dye ATTO647 was incorporated as a drug mimetic into NLC composed of Dynasan and Miglyol, produced by hot high pressure homogenization. The structures were visualized by a combination of SPT and super-resolution fluorescence microscopy based on size determination. SPT experiments were performed using a

total internal reflection fluorescence (TIRF) microscopy setup (Kim et al. 2009), to achieve single-molecule resolution. SPT analysis methods were used to determine the diffusion parameters of the drug model compounds. Data from fluorescent molecules located in a single particle revealed confined areas for their diffusion within the nanocarrier that are much smaller than the average size of the NLC itself, indicating liquid nanocompartments within the particle matrix. These nanocompartments are called super-resolution "visits maps" (Kim et al. 2012; Kirchberg et al. 2010) which with resolution down to 5 nm highlight the areas in which the dye molecules are localized within the nanocarrier. An example of these inner structures is shown in Fig. 11.13.

Phase separation was also reported by the research group of Mäder et al. (Jores et al. 2004). The separated oil phase was reported to be attached outside of the solid lipid phase forming a spoon-like structure. It should be pointed out that nanostructures are significantly influenced by the respective lipid compositions used, but it cannot be excluded that artifact formation during the microscopy sample preparation took place. As mentioned above electron microscopic investigations are limited to nonphysiological environmental conditions since the samples are investigated under vacuum after placing on a sample holder. This might result in phase transitions and shape deformation of lipid nanoparticles not present in solution or air.

11.11 Incorporation of NLC into Products

NLC can be easily incorporated into water-based dermal products (e.g., w/o creams, w/o lotions, gels) by admixing. The NLC concentrate can be added to an existing cream. However, dilution of the cream might reduce the viscosity of the formulation, when the concentrate is low viscous (e.g., 20 % NLC particles) (Müller et al. 2007). To avoid this, it is possible to produce the cream with a reduced water content, and this part of water taken out is replaced by an NLC concentrate (typically 20–45 % NLC). The concentrates are admixed by gentle stirring after production of the product and its cooling to around 30 °C. Concentrates can be obtained by cosmetic excipients suppliers, e.g., CLR GmbH Berlin and Dr. Rimpler GmbH Wedemark, both from Germany. Figure 11.14 shows an example how to calculate the required amount of NLC concentrate, in this case 20 % NLC in suspension.

In order to obtain the feature of NLC as an occlusive agent, a sufficient amount of NLC is

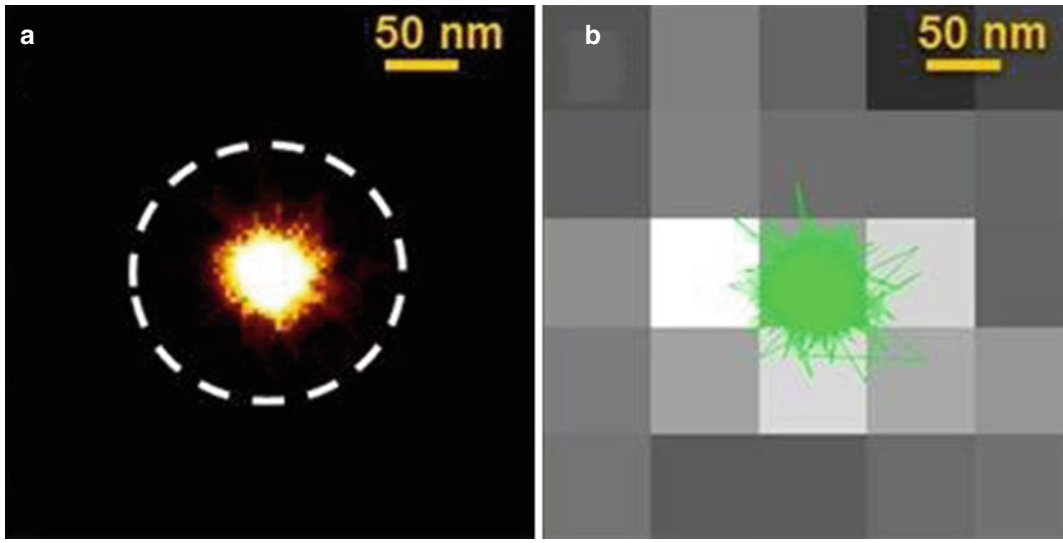

Fig. 11.13 Inner structure of NLC as determined by super-resolution (**a**) and single particle tracking (**b**) fluorescence microscopic techniques. The *white dashed circle* in (**a**) represents the outer border of the NLC

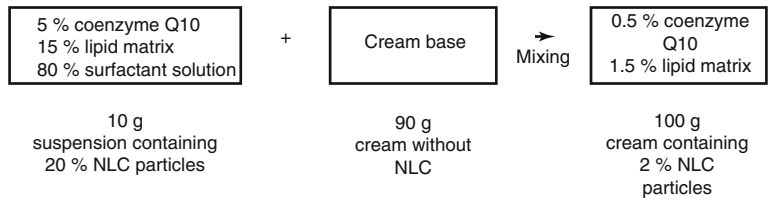

Fig. 11.14 Calculation example of admixing 20 % Q10-loaded NLC concentrate (suspension) to a water-reduced cream for a final dermal product. The NLC concentrate is diluted by a factor 10, yielding 2 % lipid nanoparticles in the final product; the final product contains 0.5 % coenzyme Q10

needed in the final formulation. Minimum concentration of NLC to provide occlusion is 2–4 %, depending on size and concentration of oil droplets present in the formulation. NLC concentrates with particles content up to 50 % (w/w) are possible to be produced and available on the market. In many cases the concentration of particles around 40 % shows already a creamy consistence. This may give some challenge to obtain a homogenous final dermal product; thorough (but gentle) mixing is therefore essential, but industrially feasible.

11.12 NLC Concentrates on the Market: Finished Products and Tailor-Made Concentrates

NLC concentrates are available with varying lipid composition (e.g., black current seed oil, hemp seed oil, kukui oil) and different actives, e.g., coenzyme Q10. Suppliers are the companies Dr. Rimpler GmbH Wedemark (http://www.rimpler.de/) and CLR GmbH Berlin (http://www.clr-berlin.de/), both from Germany. It is also possible to order a tailor-made NLC concentrate with special desired actives and oils. Development of such formulations is performed in PharmaSol GmbH Berlin (http://www.pharmasol-berlin.de/).

11.13 NLC on the Cosmetic Market: Products

The "NLC market story" is a typical example that often innovation is not driven from the large companies but small and flexible companies. The NLC story started at a meeting in the private house of the dermatologist Prof. Hans Christian Korting in Berlin, with the pharmacologist Prof. Monika Schäfer-Korting and Dr. Christian Rimpler, owner of Dr. Rimpler GmbH in 2004. In a follow-up meeting, Rimpler and Müller decided to jointly develop scientifically the first products, which were launched in October 2005.

The SLN did not make it to the cosmetic market despite their interesting features for cosmetics. The IP was owned by SkyePharma PLC (UK) (after acquisition from medac GmbH 1999), and not really exploited neither for the pharma nor for the cosmetic market. This might change now, because the SkyePharma patents expired, and the technology is now free for use.

The first two NLC products in 2005, Cutanova Cream Nanorepair Q10 and the respective serum (series NanoCare), were presented by Dr. Rimpler GmbH and Prof. R.H. Müller at the "BEAUTY FORUM" in Munich/Germany (Fig. 11.15). Meanwhile there are many products on the market by different companies worldwide, and it is too difficult really to keep an overview. Table 11.3 shows the products, which appeared on the market until 2008. The only way to get an idea of the number of present products is to estimate the tons of products which can be made from the quantity of NLC concentrates sold worldwide. However, such sales figures are kept confidential.

Meanwhile estimated more than 500 products containing NLC can be found worldwide. Often the NLCs are not listed as NLC product on the INCI nomenclature. This is possible by listing the INCI names of the actives and excipients; the particles are made from, e.g., coenzyme Q10, carnauba wax, and black current seed oil. Some

Fig. 11.15 The first two cosmetic NLC products in the world, Cutanova Cream Nanorepair Q10 (*left*) and the respective serum (series NanoCare) (*right*), presented by Dr. Rimpler GmbH and Prof. R.H. Müller at the "BEAUTY FORUM" Munich in 2005

companies advertise the use of NLC, especially in markets where nanocosmetics are popular and sold very well. Often NLCs are also used to optimize the skin feeling of products. They are added in small amounts to give unique sensation properties to a product, which can be experienced by the consumer. The product just feels different to other dermal products. The consumer should feel the difference between the NLC product and the competitor ones!

11.14 NLC in Consumer Care Products/Cosmeceuticals: Atopic Dermatitis

Consumer care products can be used to support dermatotherapy, or to reduce the need to apply pharmaceutical creams by normalizing skin function and skin condition (e.g., regarding bacterial colonization). A nice example is the silver (Ag)-NLC complex to be applied in light to medium severe atopic dermatitis (AD) (Müller et al. 2007). AD is – among others – characterized by a distorted barrier of the skin and colonization of undesired bacteria, e.g., *S. aureus*. Based on this it was logical to formulate a product containing adhesive NLC for repairing the distorted barrier by adhesion and film formation. In addition, as antimicrobial agent silver microparticles were added to the formulation to exploit the antibacterial oligodynamic effect after Nägeli (Keck and Schwabe 2009; Keck et al. 2008). From the silver microparticles, silver ions dissolve in a low concentration and act as antimicrobial.

Surprisingly it was found that a synergistic effect between the NLC and the silver ions occurred. The positively charged silver ions adsorb onto the negatively charged NLC, forming a silver-NLC complex with final positive outer charge. The Ag-loaded NLCs adsorb onto the skin, but also preferentially onto the negatively charged bacteria. This results in a high silver ion concentration locally on the bacterial membrane. This concentration on the membrane is much higher than in a cream containing just silver ions in the water phase dissolved according to the oligodynamic effect. Adsorption of the NLC complex onto bacteria enhances the Ag penetration into the bacterial membrane. This local high concentration of Ag damages the membranes and kills the bacteria (von Nägeli 1893). Figure 11.16 illustrates the mechanism. In in vitro microbiological studies, the highest killing rate for bacteria was found when incubating the complex NLC-silver microparticles which is used in the commercial product Nanosensitive (Dr. Rimpler, Germany) (Fig. 11.17).

11.15 NLC for Pharmaceutical and Cosmetic Industry: Present State and Future

Despite the convincing performance of SLN and NLC on the skin, and also their use in supportive drug therapy (e.g., atopic dermatitis), to our knowledge there are no pharmaceutical products on the market by now. Of course the question arising is: Why not?

After the above chorus of praise, this question brings the authors in the unfortunate position to explain why pharmaceutical industry did not launch products on the market when the lipid nanoparticles are such a "smashing" delivery system. Definitely we cannot take the easy way

Table 11.3 Examples of main NLC products appearing on the market between October 2005 and spring 2008

Producer/distributor and product name	Market introduction	Main active ingredients
Dr. Rimpler:		
Cutanova cream nanorepair Q10	10/2005	Coenzyme Q10, polypeptide, hibiscus extract, ginger extract, ketosugar
Intensive serum canorepair Q10	10/2005	Coenzyme Q10, polypeptide, Acmella oleracea extract
Cutanova cream nanovital Q10	06/2006	Coenzyme Q10, TiO_2, polypeptide, ursolic acid, oleanolic acid, sunflower seed extract
Chemisches Laboratorium Dr. Kurt Richter (CLR):		
NanoLipid restore CLR	04/2006	Black currant seed oil containing ω-3 and ω-6 unsaturated fatty acids
NanoLipid Q10 CLR	07/2006	Coenzyme Q10 and black current seed oil
NanoLipid basic CLR	07/2006	Caprylic/capric triglycerides (unloaded NLC)
NanoLipid repair CLR	02/2007	Black current seed oil and manuka oil
Amore Pacific:		
SuperVital Cream Serum Extra moist softener Extra moist emulsion Eye cream (line: IOPE)	09/2006	Coenzyme Q10, ω-3 and ω-6 unsaturated fatty acids
Isabelle Lancray:		
SURMER Crème Legère Nano-Protection	11/2006	Kukui nut oil, Monoi Tiare Tahiti®, coconut milk, wild indigo, pseudopeptide, noni extract
SURMER Crème Riche Nano-Restructurante	11/2006	Kukui nut oil, Monoi Tiare Tahiti®, coconut milk, wild indigo, pseudopeptide, Tamanol®
SURMER Elixir du Beauté Nano-Vitalisant	11/2006	Kukui nut oil, Monoi Tiare Tahiti®, coconut milk, pseudopeptide, wild indigo
SURMER Masque Crème Nano-Hydratant	11/2006	Kukui nut oil, Monoi Tiare Tahiti®, coconut milk, wild indigo, pseudopeptide, Tamanol®
SURMER Creme Contour Des Yeux Nano-Remodelante	03/2008	Kukui nut oil, pseudopeptide, hydrolized wheat protein, Ximenia americana seed oil, Tamanol®
Beate Johnen:		
NLC deep effect eye serum	12/2006	Coenzyme Q10, highly active oligosaccharides
NLC deep effect repair cream	12/2006	Coenzyme Q10, TiO_2, highly active oligosaccharides
NLC deep effect reconstruction cream	12/2006	Coenzyme Q10, acetyl hexapeptide-8, highly active oligosaccharides in polysaccharide matrix, micronized plant collagen
La praire:		
Swiss cellular white Illuminating eye essence	01/2007	Glycoproteins, Panax ginseng root extract, Equisetum arvense extract, Camellia sinensis leaf extract, viola tricolor extract
Swiss cellular white Intensive ampoules	01/2007	Glycoproteins, Panax ginseng root extract, Equisetum arvense extract, Camellia sinensis leaf extract, viola tricolor extract
Scholl:		
Regenerationscreme intensiv	06/2007	Macadamia ternifolia seed oil, avocado oil, urea, black current seed oil
Dr. Theiss:		
Olivenöl Anti Falten Pflegekonzentrat	02/2008	Olea europaea oil, panthenol, Acacia senegal, tocopheryl acetate
Olivenöl Augenpflegebalsam	02/2008	Olea europaea oil, Prunus amygdalus dulcis oil, hydrolized milk protein, tocopheryl acetate, Rhodiola rosea root extract, caffeine

Modified after (Müller et al. 2007; Pardeike et al. 2009). Please note: no product lists are published after 2008!

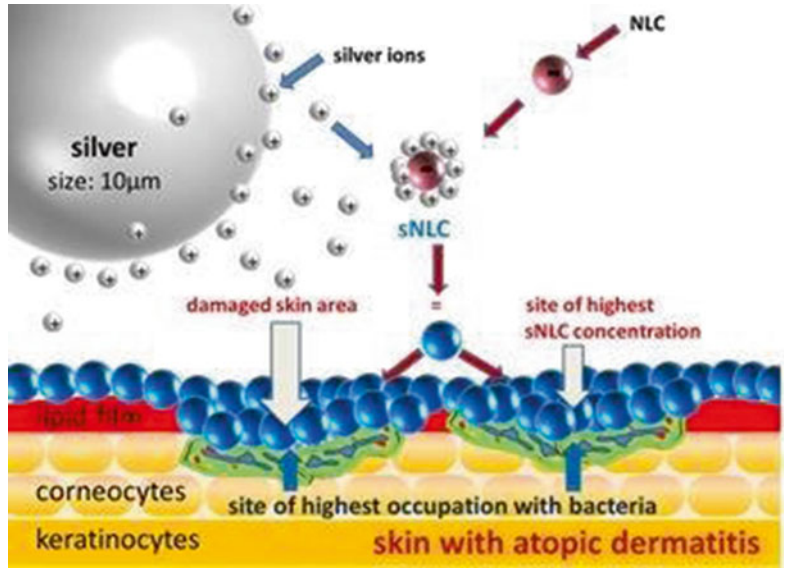

Fig. 11.16 Mechanism of action of the silver-NLC complex (explanation cf. text) (With permission after (Keck and Müller 2010))

Fig. 11.17 Product Nanosensitive cream (*left*) and lotion (*right*) (manufacturer, country) containing the silver-NLC complex, making redundant in many cases drug treatment of light to medium severe atopic dermatitis by physically repairing the distorted skin barrier in AD and by normalizing the bacterial skin colonization by optimized pure skin care – no drug treatment

out by saying: "Pharmaceutical industry was too ignorant to realize the potential in therapy & market."

There is a certain typical time between invention of a system and the introduction to the market. Since the invention of the "lipidic delivery system" – liposomes around 1965 – it took about 20 years for the liposomes to the cosmetic market (antiaging product "Capture," launched by Dior in 1986) and 25 years to the first pharmaceutical products (e.g., Alveofact by Dr. Karl Thomae GmbH, Germany). Based on this and taking 1991 as year of the invention of SLN, one could expect the first pharmaceutical products around 2016–2020.

The nanocrystals were the exception, making it to the market in the year 2000 in less than 10 years since the first patent application in 1991 (Müller et al. 2011b). However, the nanocrystals seized the opportunity. There was a drug development urgently needing this technology for market introduction, and the nanocrystals were employed for formulating and marketing this drug (sirolimus). Consequently other drugs followed this example. Identical to other developments, the lipid nanoparticles had adverse conditions in the last decade till 2010 because the industrial and financial environment changed after "nine eleven" in 2001 and after the financial crisis in autumn 2008. Companies rethought their investments and product development policy and turned to be more conservative and more reluctant in new investments. To our knowledge, e.g., clinical testing of one dermal lipid nanoparticle product was not entered due to the financial crisis in 2008.

Meanwhile developments toward the clinic started again. It is expected that the first SLN or NLC pharmaceutical product will be either a dermal or an oral product. In dermal it is expected that primarily new molecules will be in the focus exhibiting penetration problems, or an "old" molecule within life cycle management, to deal with upcoming competing generic products. However, most likely no company will replace a well selling existing dermal product by an NLC product providing some advantages, because the development costs will not be recovered. Apart from dermal, NLCs possess also big potential in oral delivery, because lipids promote the oral absorption of many drugs. Absorption is especially pronounced, when the drug is associated with a lipid (= dissolved in lipid), and the lipid is finely dispersed – both perfectly achieved in lipid nanoparticles. It is also an option to complement dermal cosmetic products by an oral product (e.g., coenzyme Q10 cream plus Q10 NLC capsules – "beauty from outside and inside").

11.16 Conclusions and Perspectives

The lipid nanoparticles (SLN) with solid particle matrix were developed by two research groups in parallel, filed as patent applications in 1991. The scientific community voted not with one's feet but with its hands and brains: Many research groups worldwide started working with this system, judging it as a very promising system for controlled drug delivery for different application routes, dermal and also oral, full name (i.v.) and parenteral in general.

The number of research groups is clearly documented by the published articles. The key word "solid lipid nanoparticles" put into Google gives 1.49 million hits (on the 13th May 2013). It is 4.32 million hits for "polymeric nanoparticles," but the pharmaceutical polymeric nanoparticles were invented in 1973 by P.P. Speiser and are now 40 years old; the lipid nanoparticles are a just grown up 21-year-old system. Similar to liposomes the lipid nanoparticles in the form of NLC have entered the cosmetic market. Taking – for being correctly – the filing year of SLN patent applications, it took them only 15 years to the worldwide cosmetic market, faster than the liposomes. Product-make-ability is proven, and large industrial scale production is confirmed (or is reality?). Based on this, the lipid nanoparticles (SLN, NLC) should have a fair chance to make it also to the pharmaceutical market – hopefully within the next decade improving dermal and/or oral delivery.

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12 Polymeric Nano (and Micro) Particles as Carriers for Enhanced Skin Penetration

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12.1 Introduction

The skin is one of the largest body organs in surface area and weight which makes it an excellent candidate for drug delivery purposes. Application of drugs to the skin could have a direct effect on the skin and skin structures (topical drug delivery) or achieve a systemic effect after absorption in the systemic circulation (transdermal drug delivery). Since the main function of the skin is protective and it is acting as a main barrier against external toxins and invading infectious microorganisms as well as preventing the loss of physiologically essential substances as water, skin is known to be relatively impermeable. The main barrier properties are presented by the uppermost layer of the skin which is the stratum corneum (SC).

In both cases of topical and transdermal applications, the drug needs to cross the SC barrier. The difference is that in case of transdermal applications, the flux through the skin to the general circulation needs to be maximized with minimum skin retention and vice versa for the topical applications. Transdermal drug delivery is becoming more attractive alternative to other drug delivery routes due to several perceived advantages including the safe application with minimum injury, controlled drug delivery, ease of termination of therapy, higher patient compliance, avoiding first-pass metabolism, and reduced side effects. Efforts are always made to enhance the penetration of therapeutically active materials

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through the skin by different chemical and physical methods. Physical methods include iontophoresis, electroporation, ultrasound, microporation, radiofrequency, and microneedles to open up the skin (Patel et al. 2010). Chemical methods include the use of organic solvents or compounds that help to increase the partitioning of the drug to skin structures or disrupt the highly organized lipid structure of the SC, thus increasing drug diffusivity into the skin (Pathan and Setty 2009).

Over the past few decades, the use of colloidal systems for drug delivery to the skin has been extensively explored as an alternative to the classic chemical penetration enhancer systems. Because of the small size of such systems, they are thought to have special properties like higher reactivity and unique interactions with biological systems leading to better pharmacokinetics and therapeutic indices of the encapsulated drugs (Zhang et al. 2010). They can easily be saturated with the loaded drug leading to higher permeation enhancement compared to simple vehicles where the drug is subsaturated (Davis et al. 2002). Wide range of polymeric micro- and nanocarriers were developed for skin applications offering several advantages in systemic drug delivery like controlled drug release as well as in local skin therapy by achieving targeted drug delivery to certain skin layers or appendages (Tallau et al. 2010). Polymeric nanoparticles also offer higher stability compared to other vesicular systems like liposomes, niosomes, and ethosomes. In addition they provide protection to labile compounds against premature degradation and reduce skin irritation (Batheja et al. 2011).

The aim of this chapter is to discuss the use of polymeric micro- and nanoparticles for enhanced percutaneous absorption of drugs and their potential to be used as transdermal drug delivery systems.

12.2 Polymeric Microparticles for Enhanced Skin Penetration

Polymeric microspheres were proposed as candidates for follicular targeting of drugs since they possess several advantages including high stability on the skin, ease of preparation, ability to supply protection to the encapsulated drug, controlled drug release, and the possibility to incorporate both hydrophilic and lipophilic drugs (Rolland et al. 1993). Due to the high impermeability and the strong barrier properties of the skin, hair follicles have been always seen as an interruption traps which can give a way to drugs that are not able to penetrate the skin through transcellular pathway. The hair follicle infundibulum is a further region of interest because it is surrounded by a high density of immune cells and intensive capillary network, thus enhancing the potential for topical vaccination and transfollicular absorption to the systemic circulation (Patzelt et al. 2011; Vogt et al. 2008).

One of the first reports on microparticles was the use of 5 μm adapalene-loaded microspheres for the site-specific treatment of acne which led to a promising targeting strategy as evidenced from follicular biopsies of human skin (Rolland et al. 1993). On the other side, 1 μm particles were found randomly scattered in the stratum corneum and follicular ducts, while 20 μm microspheres were not able to penetrate the skin and remained on the surface. It is to be mentioned that 3 min of massage preceded testing the penetration behavior of these microspheres. 5 μm rhodamine-loaded silicone microspheres were then used as a tool to enhance the follicular penetration of a fluorescent dye to increase the efficiency of laser hair removal (Sumian et al. 1999). In another study, looking for the optimum size for follicular targeting, the optimum size was found to be around 1.5 μm combined with cyanoacrylate skin surface stripping technique to enhance the microsphere penetration (Toll et al. 2004). In the above studies, it can be seen that external forces either massage or skin stripping were required to enhance the accumulation of microparticles into the follicular openings. Distribution of poly(D,L-lactic–co-glycolic acid) (PLGA) microparticles in porcine skin after its topical application was studied in vitro using microparticles containing rhodamine as a fluorescent probe. The biodegradable PLGA microparticles were prepared using w/o solvent evaporation technique using polyvinyl alcohol as a stabilizer

in the size range of 1–10 μm with an average volume diameter of 4.61 μm. Permeation experiments and microscopical examination revealed that the microparticles could be detected in the SC and the upper layers of epidermis but not in the dermis or in the receptor compartments (de Jalon et al. 2001a). On the other hand, application of particles ranging in the size from 122 to 1000 nm to porcine ear skin revealed that PLGA particles and silica particles in the size of 643 and 646 nm, respectively, had the deepest penetration in hair follicles compared to smaller and larger particles (Patzelt et al. 2011). Application of ethylcellulose nanoparticles ranging in size from 50 to 1000 nm revealed insignificant accumulation in hair follicles of healthy mice skin, regardless of the particle size (Abdel-Mottaleb et al. 2012a). As seen, there are large differences in the results of these studies, making it very hard to generalize. This might be because of the different animal models utilized, application procedures, and properties of the particles, confirming that further are needed to fully understand the micro- or nanoparticle-mediated transfollicular drug delivery (Lu et al. 2008).

Examples on the use of polymeric microparticles for dermal or transdermal applications include titanium dioxide (TiO_2)-coated microparticles which are commonly used as UV filters in commercial sunscreen products. Even after repetitive application, penetration of the microparticles into viable skin tissue could not be detected. Only on the horny layer of the SC and in the orifices of hair follicular openings microparticles could be identified (Lademann et al. 1999). The use of PLGA microparticles loaded with the antiviral drug acyclovir was explored as a tool to enhance its intraepidermal concentration and to achieve sustained drug release compared to drug suspension. The amount of the drug detected in the receptor compartment was much lower upon using microparticles, while drug concentration in the dermis was higher than suspension indicating that the microparticulate drug delivery system was suitable for the topical drug delivery of acyclovir (de Jalon et al. 2001b). In another work, the antiviral drug cidofovir was encapsulated in poly (lactic acid) PLA/PLGA microparticles. Skin permeation experiments showed higher accumulation of the drug in the upper skin layers from the microparticles compared to drug solution, while the skin permeation of the drug was higher from solution than microparticles (5.27 versus 3.36 μg/cm^{2}h). Therefore, authors suggested that the use of microparticles could improve the treatment of skin viral infections with minimal side effects (Santoyo et al. 2002). Collagen microparticles were used to enhance the dermal delivery of all-trans retinol and to increase its stability where its sorption onto the microparticles prevented retinol crystallization. The retinol microparticles were suspended into hydrogels and compared to hydrogels containing the free drug. Hydrogels containing retinol adsorbed on collagen microparticles achieved faster and higher transport in different skin layers of hairless mice in vitro compared to hydrogels with free drug (Rossler et al. 1994).

An interesting application was the use of microparticles to increase the homogeneity of the distribution of protection creams on the surface of the skin to form a homogenous film upon drying. Creams containing antioxidants against palmar-plantar erythema caused by the chemotherapeutic agent doxorubicin, that is, excreted in sweat, were found to have higher efficiency when microparticles in the size range of 10–100 μm were incorporated. This property is also very important for sunscreens and any film forming topically applied formulations (Lademann et al. 2008).

The skin, although a potent immunological induction site, is rarely used for vaccination because of its poor permeability to topically applied vaccines and fair accessibility by needles (Chen et al. 2000). Ballistic drug delivery is a needle-free technique in which vaccines and drugs formulated as microparticles are sufficiently accelerated to penetrate the outer layer of the skin and achieve a pharmacological effect. The device used for shooting the particles is called gene gun which accelerates the microparticles while producing minimal damage to the targeted cells (Rinberg et al. 2005). These particles could be in the size range of 10–20 μm, but more recently DNA vaccination started to be

more applied where the smaller gold particles in the range of 1–2 μm coated with a DNA construct are targeted to immunologically sensitive cells in the epidermis (Kendall et al. 2004). Several parameters related to the particle size, density, and injection speed have to be optimized in order to enhance transfection efficiency and minimize tissue damage. For example, increasing humidity or temperature of the skin decreases the stiffness of the stratum corneum and subsequently increases the penetration of the microparticles deeply into the skin and brings the drug closer to the cutaneous blood capillaries (Cevc and Vierl 2010). The costs and complexity of gene guns limit their clinical use in human vaccination; however, there are several alternative devices used for epidermal particle-mediated immunization that are reviewed elsewhere (Kis et al. 2012). After intradermal injection of peptide-loaded PLGA microparticles prepared by the double emulsion solvent extraction technique, powerful antibody and T-cell responses were obtained compared to the conventional subcutaneous injections even with very low doses of the antigen. The antigen used was SPf66 antigen which is a malaria synthetic peptide vaccine (Carcaboso et al. 2004). The microencapsulation enhanced the targeting efficiency of the peptide to the dendritic cells in the skin, thus embracing the skin as an immune organ. Therefore, microparticles can be considered efficient carriers for topical skin application, follicular targeting, and immunological or vaccination purposes, while their potential to enhance percutaneous permeation is rather negligible.

12.3 Polymeric Nanoparticles as Drug Carrier

Polymeric nanoparticles have been defined in the pharmaceutical environment as colloidal particles ranging in size from 10 to 1000 nm and are generally made of polymers that may be natural or synthetic. The definition of the term "nanoparticles" is still a matter of debate that some societies prefer to define them as less than 100 nm in size (Zhang et al. 2010), others say favorably smaller than 500 nm (Couvreur 2013), but by all ways they are not larger than 1 μm in size. The use of nanoparticles for drug delivery will depend on their bio-acceptability that is in turn affected by the physicochemical properties of the polymer and particle size. Therefore, the therapeutic goal will define the final choice of polymer used, particle size, candidate drugs, and manufacturing methods. Depending on the preparation technique, nanospheres (matrix-type nanoparticles) or nanocapsules (reservoir-type nanoparticles) can be obtained (Tallau et al. 2010). For pharmaceutical applications, drugs can be entrapped, dispersed, dissolved within, or adsorbed on the nanoparticles as seen in Fig. 12.1 (Guterres et al. 2007).

Depending on the nature of the nanoparticles' structure, polymer used, and the physicochemical properties of the drug loaded, drug release can take place by either one mechanism or a combination of different mechanisms. Desorption of the drug from the nanoparticles surface can take place in both nanospheres and nanocapsules. Drug diffusion through the polymer matrix of nanospheres or polymer shell of nanocapsules as well as polymer matrix erosion in nanospheres are other possible mechanisms (Cosco et al. 2008). Initial burst drug release is always common from polymeric nanoparticles as a result of the immediate desorption of the amount of drug adsorbed on particles' surfaces. Therefore, faster release is always expected if the drug is solely adsorbed on the surface of nanospheres or nanocapsules without any internal encapsulation as in the case of most of the hydrophilic drugs. For lipophilic drugs which can be homogenously dissolved or distributed in the polymer matrix, release will occur mainly by diffusion through the polymer matrix or by erosion. The predominant mechanism will be determined whether drug diffusion is faster than polymer degradation or not (Soppimath et al. 2001). Drug loading and its physicochemical properties have also great role in determining the mechanism of drug release. Drug release from PLA nanospheres loaded with different concentrations of lidocaine (7–32 % w/w) was studied (Polakovic et al. 1999). When drug concentration was below 10 %, the drug was

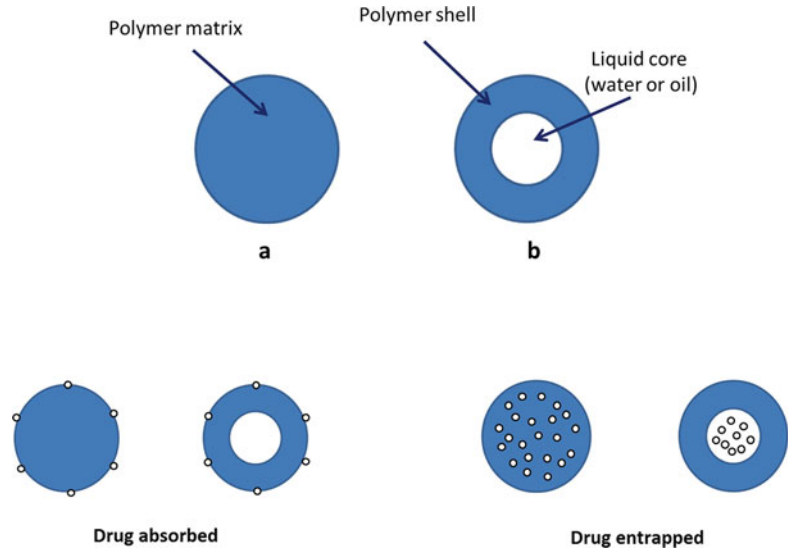

Fig. 12.1 Schematic representation of polymeric nanospheres (**a**) and nanocapsules (**b**) with drugs either adsorbed on the surface or entrapped in their core

molecularly dispersed in the polymer matrix and the release followed a diffusion model. At higher concentrations, solid lidocaine crystals existed and the drug had to dissolve first and to diffuse through the polymer matrix, and the overall release mechanism was by dissolution. Chitosan nanoparticles loaded with hydrocortisone were prepared by ionic cross-linking of chitosan with tri-poly-phosphate (Katas et al. 2012). Drug release was controlled mainly by the swelling of the polymer which could be modified by the polymer molecular weight, degree of cross-linking, and pH of the dissolution medium.

Various biodegradable polymers attracted attention for controlled delivery of low molecular weight drugs as well as bioactive proteins (Park et al. 2005). Both synthetic and natural biodegradable polymers have been investigated as drug delivery systems. They are characterized by the presence of hydrolytically or enzymatically sensitive bonds in the polymer leading to polymer erosion into biocompatible by-products (Katti et al. 2002). Natural polymers usually undergo enzymatic degradation, while synthetic polymers are mostly hydrolytically degradable which makes them less sensitive to site to site or patient to patient variation. Synthetic polymers have lower biological activity compared to natural ones, have less batch to batch variation, and can be tailored to match different applications (Nair and Laurencin 2007). Polylactides and polyglycolides are the most commonly used biodegradable polyester polymers. Because of the very slow degradation rate of the first and the fast degradation of the latter, copolymers of them (poly lactide-co-glycolide) have been developed with various ratios and are used in wide range of biomedical applications. Polyanhydrides, polycaprolactone, polydioxane, and poly(alkyl cyanoacrylates) are other examples of synthetic biodegradable polymers. Natural enzymatically degradable polymers such as hyaluronic acid, collagen, polyglutamic acid, chitosan, and alginic acid are also extensively used for drug delivery applications.

Multiple preparation techniques exist for the production of polymeric nanoparticles. These methods may utilize a preformed polymer or may depend on the in situ polymerization of monomers during the preparation of nanoparticles. Choice of the preparation technique depends on several factors including the type of polymer used, intended purpose or use, and required particle size. In situ polymerization of monomers can be achieved by emulsion polymerization, interfacial polymerization, and controlled radical polymerization. Tailored nanoparticles with certain desired properties can be produced by the polymerization of monomers carrying these properties. Emulsion polymerization is the most commonly

used method where the monomers, initiators, and surfactants are all mixed in water and the polymerization reaction results in solid particle formation (Lu et al. 2009). Interfacial polymerization is another well-established technique for the production of nanoparticles in which polymerization of two reactive monomers dissolved in two different phases takes place at the interface. It is a common technique for the production of polymeric nanocapsules containing water or oil where the reaction takes place on droplets' surfaces of water/oil or oil/water microemulsions, respectively (Khoury-Fallouh et al. 1986; Watnasirichaikul et al. 2000). With the exception of alkyl-cyanoacrylates and dialkyl-methylidene malonate, most of the monomers suitable for in situ polymerization process lead to very slowly biodegradable or nonbiodegradable polymers. In addition, extensive purification procedures for the removal of residual molecules including monomers, oligomers, surfactants, and initiators which might have toxic potential are required (Pinto Reis et al. 2006). Therefore, methods utilizing preformed polymers have been proposed. Methods utilizing preformed polymer dispersion include solvent evaporation, salting out, nanoprecipitation, dialysis, and supercritical fluid technology (Rao and Geckeler 2011).

Solvent evaporation techniques involve the solubilization of polymers in organic solvents such as dichloromethane and chloroform and preferably ethyl acetate, which has a lower toxic potential. The organic polymer solution is then emulsified in aqueous surfactant solution, and evaporation of the solvent is performed under reduced pressure or by continuous magnetic stirring yielding nanoparticle suspension (Allemann et al. 1993). The method is suitable for the encapsulation of lipophilic drugs as they are easily dissolved in the organic polymer solution. In case of hydrophilic ingredients, double emulsion techniques (water/oil/water) can be utilized. The use of salting out for nanoparticles' formulation includes dissolving the polymer in water miscible solvent, mainly acetone, followed by emulsification in aqueous electrolyte surfactant solution. Solvent extraction is then performed by adding excess water to allow complete diffusion of acetone into the aqueous phase resulting in the formation of nanoparticles (Allemann et al. 1992). Nanoprecipitation, known also as solvent displacement method, depends on the interfacial deposition of the polymer during the slow addition of organic polymer solution to an aqueous phase under moderate stirring (Fessi et al. 1989). It is a fast, reproducible, and easy technique for the preparation of nanoparticles, but low polymer concentration in the organic phase is a major drawback (Rao and Geckeler 2011). The dialysis technique is performed by dialyzing organic polymer solution against water where solvent displacement leads to reduced polymer solubility and results in a homogenous suspension of nanoparticles. Recently, supercritical fluid technology has been proposed as a preparation technique to avoid the use of risky organic solvents and produce particles of high purity (Meziani et al. 2004). Precise control of the experimental parameters has to be ensured to produce nanoscale particles rather that microparticles, and the technique is also limited because of the poor solubility of most polymers in the supercritical fluids.

12.4 Polymeric Nanoparticles for Enhanced Skin Penetration

Polymeric nanoparticles possess several unique characteristics for skin drug delivery, as their size, zeta potential, and drug release profiles could be specifically controlled by using different polymer lengths, surfactants, and preparation techniques. It was evidently believed that polymeric nanoparticles have a potential to alter the activity of dermally applied drugs by changing the rate of their release and increasing drug residence time in the skin. A sustained drug release might reduce systemic drug absorption and reduce the systemic side effects. This makes them excellent candidates for topical application of drugs intended to have a localized effects on skin diseases (Abdel-Mottaleb et al. 2011). Therefore, there are only limited numbers of investigations supporting the transdermal drug

permeation-enhancing effect of polymeric nanoparticles.

Investigation of the influence of nanoencapsulation of the anti-inflammatory drug flufenamic acid on its skin penetration and permeation revealed that its incorporation into biodegradable PLGA nanoparticles led to slight increase in the drug uptake especially after long incubation times (Luengo et al. 2006). For short incubation times, the drug permeation and penetration was found to be higher for the free drug compared to the nanoparticles, which proves that nanoencapsulation could provide sustained release of the drug more than permeation-enhancing effect. The use of biodegradable poly (ϵ-caprolactone) nanoparticles to enhance passive transport of the encapsulated highly lipophilic sunscreen octyl methoxycinnamate (OMC) was investigated, and it was found that higher concentrations of the drug can be detected in the stratum corneum, but without any detectable permeation through the skin compared to OMC emulsion. This is owed to that molecules encapsulated in nanoparticles may have a higher thermodynamic activity compared to a solution which facilitates their partitioning into the skin (Alvarez-Roman et al. 2004). However, if the drug is highly lipophilic, this will lead only to its accumulation into the stratum corneum without any further penetration. Stratum corneum penetration of OMC formulated as nanocapsules was also compared to conventional w/o emulsion and nanoemulsion (Olvera-Martinez et al. 2005). Due to the small size and flexibility of nanoemulsion droplets, they had a higher penetration rate compared to the relatively larger rigid polymeric nanocapsules.

Diblock copolymer of poly(ϵ-caprolactone)-block-poly(ethylene glycol) was synthesized to prepare self-assembled minoxidil-loaded polymer nanoparticles of two size ranges, 40 and 130 nm. In hairy guinea pig skin, minoxidil permeation from the 40 nm particles was 1.7-fold higher in the receptor solution than that of 130 nm particles. In hairless guinea pig skin, no significant difference was detected between the two formulations, suggesting that the smaller nanoparticles could enhance the drug permeation through shunt ways (Shim et al. 2004).

Ethylcellulose and PLGA nanoparticles were tested for their permeation-enhancing effect of the anti-inflammatory drug ibuprofen. Although the permeation of ibuprofen from polymeric nanoparticles was higher than from drug solution, the permeation-enhancing effect of other lipid-based systems including lipid nanocapsules, solid lipid nanoparticles, and nanostructured lipid carriers was much higher than that of polymeric nanoparticles and more than four times higher than that of the drug solution (Abdel-Mottaleb et al. 2011).

Nanospheres produced from tyrosine-derived triblock copolymer have been proposed as an effective permeation enhancer for lipophilic compounds into lower skin strata. They proved to be biocompatible and safe topical delivery system as they did not induce any short-term cytotoxicity or morphological changes in the stratum corneum. However, the encapsulated model lipophilic compound "Nile red" could not be detected in the receptor compartment confirming the ability of polymeric nanospheres to enhance the residence of the drug in the skin without increasing its transdermal permeation (Batheja et al. 2011).

Poly(n-butyl cyanoacrylate) (PNBCA) nanocapsules prepared by interfacial polymerization were investigated for the transdermal application of indomethacin (Miyazaki et al. 2003). In vitro skin permeation experiments showed that the incorporation of indomethacin into the nanocapsules could significantly enhance its skin permeation compared to free drug, and slower controlled release could be achieved by their incorporation into poloxamer 407 (Pluronic® F-127) gels. In vivo experiments also showed higher plasma levels and an increase in the AUC of indomethacin from the nanocapsules by a factor of 3.3 compared to the poloxamer 407 (Pluronic® F-127) gel formulation. The authors suggested that the in vitro rate of drug release from the nanocapsules was not sufficient to account for the total appearance of the drug in the acceptor compartment indicating the permeation of intact nanocapsules through the skin. However, further studies were needed to confirm this hypothesis. On the other side, a similar study utilized poly(e-caprolactone) nanocapsules loaded with chlorhexidine. In this case,

drug permeation and release data were highly correlated, suggesting that the percutaneous absorption was solely controlled by the diffusion across the polymeric barrier (Lboutounne et al. 2004). PLGA and chitosan bilayered nanoparticles containing ketoprofen and spantide II, surface modified with oleic acid were used to enhance drug delivery to deeper skin layers for the treatment of dermatitis and psoriasis (Shah et al. 2012). The particles were prepared by modified solvent evaporation technique where the PLGA nanoparticles were formed with an outer layer of chitosan which was then cross-linked to form bilayered nanoparticles. Skin permeation studies showed non-detectable amounts of spantide in the receptor compartment, while its retention in various skin layers was increased approximately 5.7 and 3.7 times compared to solution or non-surface-modified nanoparticles, respectively. In case of ketoprofen, skin retention was also increased by 6.8- and 4.1-fold compared to drug solution and surface nonmodified particles. In contrary to spantide, ketoprofen was detected in the receptor compartment, and its permeation was highest from the bilayered nanoparticles without oleic acid modification. The permeated amount from these nanoparticles was 7 and 3.2 times higher than from ketoprofen solution and oleic acid-modified nanoparticles, respectively. This indicated that surface modification led to improved skin retention by first binding to the stratum corneum due to their net positive charge. As the time progresses, water from the nanoparticles evaporates and forms a thin film that enhances skin hydration, and then oleic acid as a penetration enhancer starts to disturb the lipid domains of SC leading to deeper permeation of the loaded drugs. However, these polymeric nanoparticles were also found to be better suited for localized treatment of skin diseases, as the skin permeation enhancement was limited.

Polymeric nanoparticles have been also proposed for the vaccination purposes as non-viral gene delivery systems. Chitosan nanoparticles containing plasmid deoxyribonucleic acid (DNA) were used for immunization by topical skin application on shaved Balb/C mice, and the immunization potential was much higher compared to mice immunized with naked plasmid DNA. Moreover, immunoglobulin G (IgG) titres were even comparable to mice injected with intramuscular plasmid DNA (Cui and Mumper 2001). Transcutaneous immunization with the immunogen ovalbumin loaded into 150 nm PLA nanoparticles was also reported (Mattheolabakis et al. 2010). Higher levels of gamma interferon (INF-ɣ) and interleukin-2 (IL-2) were obtained in comparison to the ovalbumin solution due to higher uptake of the encapsulated antigen by the antigen-presenting cells in the follicular ducts of the skin. These results indicated that as far as the cellular response is concerned, it was much advantageous to deliver the antigen in nanoencapsulated form where it could be protected until it is taken up by the cells and then released inside the cells.

Because of the advances in nanoparticle engineering, formulation sciences, and improved understanding of particulate carrier systems, skin interactions will undoubtedly lead to significant progress in their topical and transdermal drug delivery applications. Reviewing the literature provides a general conclusion that micro- and nanoparticles' skin penetration is negligible (Roberts 2006; Prow et al. 2011). However, this can be considered definitely true for the healthy skin, while there is an evident lack of data or information concerning the penetration of particulate carriers in diseased skin where the barrier function may be impaired. This is considered a basic deficiency in the possibility to apply our knowledge to the enhancement of dermal and transdermal drug delivery.

Recently, there are several studies dealt with the application of nanotechnology in the treatment of different skin disorders and the basis for the proper selection of suitable nanocarrier systems for different dermal and transdermal applications. The results indicated that lipid nanocapsules were best suited to transdermal drug delivery with minimum skin retention, while solid lipid nanoparticles had both penetration-enhancing effects and high localized effects (Abdel-Mottaleb et al. 2011). However, polymeric nanoparticles showed higher tendency

to be retained on the skin surface and release the drug slowly into the skin structures without any deeper penetration though the skin as seen in Fig. 12.2.

Since a main research interest was focused on the treatment of inflammatory disease, the penetration of polymeric nanoparticles in inflamed skin was another major concern. In the case of healthy skin, it was found that all the particles were retained on the skin surface with some preferential accumulation in skin furrows, hair follicle openings, and hairs. On the other hand, in inflamed skin it became visible that larger particles (1000 and 500 nm) were also retained on the skin surface without significant differences in comparison to their behavior in healthy skin, while smaller particles (50 and 100 nm) could accumulate deeply in the hair follicles around hair shafts and in the associated sebaceous glands (Fig. 12.3). The potential changes in the skin structure associated with the inflammation process must be responsible for this enhanced and selective accumulation of nanoparticles of the small sizes into the skin. Increased accumulation of intercellular fluid in the epidermis can physically pull the keratinocytes away from each other and allows for higher penetration of the particles. On cellular level, it is known that sebaceous glands and hair follicles have abundant concentrations of immunologically active Langerhans cells, macrophages, and monocytes that might be able to internalize only nanoparticles of small sizes. Superior therapeutic efficiency of the small 100 nm betamethasone-loaded nanoparticles was also obtained in comparison to 1 μm particles (Abdel-Mottaleb et al. 2012a). The influence of nanoparticles on the systemic availability of the drug in the case of inflamed skin is not yet completely clear.

The use of charged particles has been proposed as a strategy to enhance the penetration of drugs through the SC. Positively charged particles were found to have higher deposition on porcine skin and delivered higher amounts of the loaded fluorophore N-(2,6-diisopropylphenyl) perylene-3,4-dicarboximine compared to the anionic nanoparticles (Wu et al. 2010). This might be due to the net negative charge of the skin at physiological conditions that allows higher permeation of the cationic species (Marro et al. 2001). Transcutaneous immunization with plasmid DNA coated on cationic PLGA nanoparticles could potentially induce stronger immune response than the negatively charged ones (Kumar et al. 2012). Other example of the successful use of positively charged nanoparticles for skin application was the chitosan-lecithin nanoparticles loaded with quercetin, where its skin permeation was greatly enhanced due to the significant interaction of nanoparticles with the SC components (Tan et al. 2011). Other contradictory studies showed that the application of negatively charged particles in two different sizes, 50 and 500 nm, was able to permeate porcine skin while positively charged and neutral particles could not. This was explained by the repulsion forces between the negatively charged skin and the negative particles that generate tem-

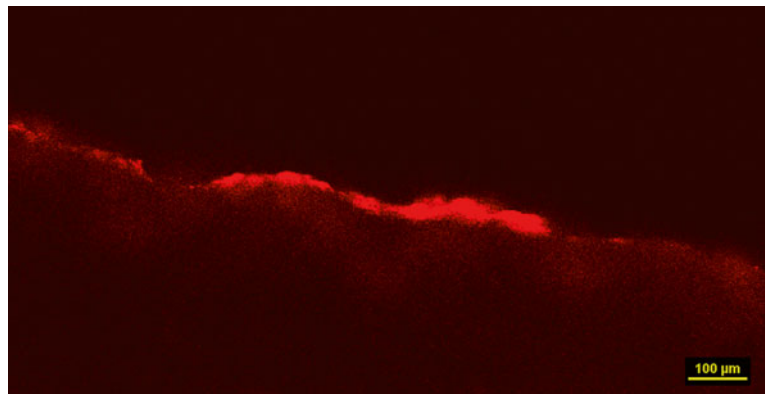

Fig. 12.2 Confocal laser scanning microscopy (CLSM) image showing side view of porcine skin treated with Nile red-loaded PLGA nanoparticles (50 nm) after 8-h incubation. The picture shows that the particles remained on the surface of the skin without any deeper penetration in different skin layers

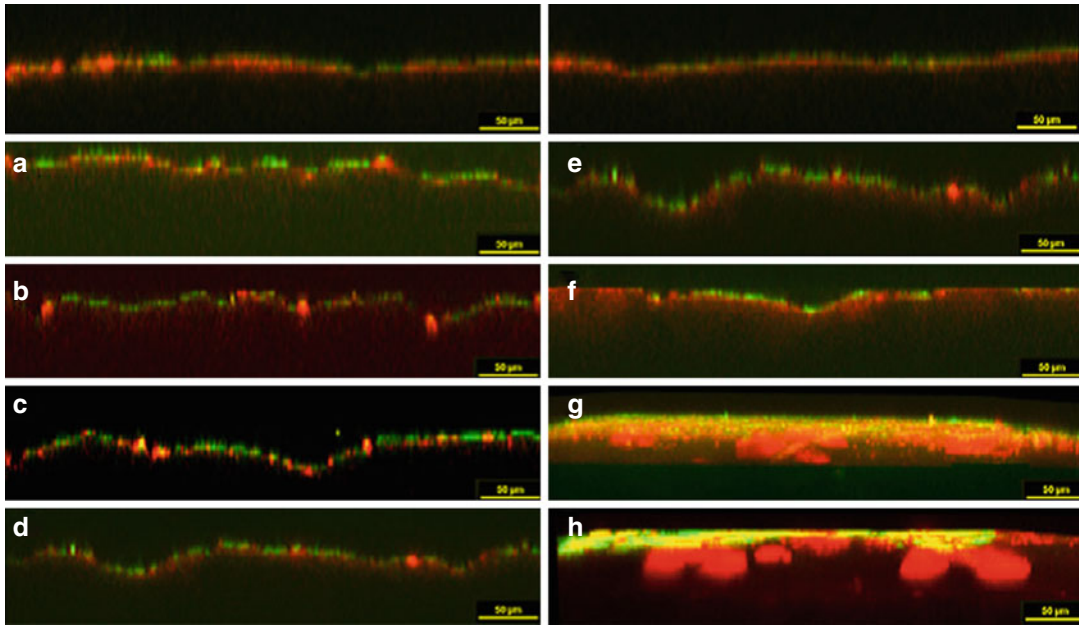

Fig. 12.3 CLSM images showing side view of skin treated with Nile red-loaded nanoparticles (NP) of different sizes (50–1000 nm) showing the depth of penetration. (**a**–**d**) Healthy skin treated with NP1000, NP500, NP100, and NP50, respectively. (**e**–**h**) Inflamed skin treated with NP 1000, NP500, NP100, and NP50. The first row is control experiment representing the healthy skin (*up left*) and inflamed skin (*up right*) treated with Nile red oily solution (Abdel-Mottaleb et al. 2012a)

porary channels in the skin, allowing for particle permeation (Kohli and Alpar 2004). The negatively charged hydrocortisone-loaded poly e-caprolactone nanoparticles were very effective in the fast control of atopic dermatitis and avoidance of corticosteroids' long-term side effects by increasing the efficiency of the drug and enhancing its skin concentration (Rosado et al. 2013). Penetration of ethylcellulose nanoparticles in size range around 100 nm with different surface charges (neutral, negative, and positive) was similar in healthy mice skin and was limited to the surface of the SC layer (Abdel-Mottaleb et al. 2012b). This finding is not in line with previous observations which may be due to the different experimental setup where this experiment was carried out on mice skin in vivo, while the preceding ones were ex vivo porcine skin permeation experiments. However, charged particles were found to achieve higher therapeutic effect when betamethasone-loaded nanoparticles carrying either negative or positive charge were compared to neutral nanoparticles in the treatment of dermatitis. This shows that the surface-charged nanoparticles carry a higher potential for dermal and transdermal drug delivery purposes compared to neutral particles.

Finally to summarize, it is evident from the reviewed studies here that polymeric nanoparticles are efficient carriers for topical treatment of skin diseases. Due to their rigid nature and significant stability, they are retained in the skin and release the drug in a sustained manner resulting in higher efficacy of localized therapy with minimum systemic side effects. In an indirect way this hypothesis is also supported by the lack of ability of intact polymeric nanoparticles to penetrate the skin. However, to determine if they hold promise as carriers for transdermal drug delivery and enhanced skin permeation, additional investigations are required. Several findings revealed that drug permeation from polymeric nanoparticles after 8 h is low in comparison to conventional creams and lipid-based nanocarriers. Additional investigations for longer times or in vivo pharmacokinetic analysis should be per-

formed in order to confirm the domination of the local delivery or to clarify if there is a tendency to enhance drug permeation through the skin over longer periods of time and the possibility of using polymeric nanoparticles for sustained transdermal drug delivery applications.

Conclusion

Polymeric micro- and nanoparticles, especially those produced from biodegradable polymers, hold promise as drug delivery systems for skin application. Due to their rigid polymeric structures, they have higher tendency to enhance skin retention and provide sustained release of drugs into different skin layers and structures rather than enhanced skin permeation to the systemic circulation. Therefore, they are better suited for topical treatment of skin diseases while they show limited advantages as transdermal drug delivery systems. Subsequently, promising findings about the selective and enhanced accumulation of such particles in diseased skin, such as dermatitis, underline rather the targeting potential of polymer particles that enhance their therapeutic efficiency at local level and reduce the associated side effects than the conventional drug delivery systems.

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Polymeric Nanocapsules for Topical Delivery

13

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13.1 Introduction

Polymeric nanocapsules have received increasing interest for topical delivery of drugs or active substances mainly for the treatment of skin diseases and skin care. The nanocapsule can be defined as a nano-vesicular system composed of an oily core surrounded by a polymeric wall (Guterres et al. 2007; Mora-Huertas et al. 2010). The studies concerning the influence of polymeric nanocapsules on the skin penetration/permeation of active substances are recent; the first study was carried out in the early 2000s. Nevertheless, the number of peer-reviewed articles increased significantly in the last few years. Fifty percent of the documents were published in the last 2 years (Web of Science®, Science Citation Index Expanded database).

Nanocapsules have important advantages for topical application such as the improvement of the stability of active substances, the reduction of their irritant effects, and their sustained release into the skin (Alvarez-Román et al. 2001; Fontana et al. 2009; Paese et al. 2009; Contri et al. 2010). Depending on the active substance, nanocapsules can improve or limit their penetration/permeation through the skin. Most studies have demonstrated that the nanocapsules are able to increase the concentration of active substances in the outermost layers of the skin (Lboutounne et al. 2002; Alvarez-Román et al. 2004; Jiménez et al. 2004; Olvera-Martínez et al. 2005; Calderilla-Fajardo

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et al. 2006; Vettor et al. 2010; Weiss-Angeli et al. 2010; Ourique et al. 2011; Siqueira et al. 2011; Detoni et al. 2012; Hanno et al. 2012; Hoffmeister et al. 2012). This is an eligible effect for active substances that have their site of action restricted to the skin surface or to the first layers of the skin, such as sunscreens (Weiss-Angeli et al. 2010), retinoids (Ourique et al. 2011), and antiseptics (Lboutounne et al. 2002). In this case, the aim is to restrain or avoid the systemic absorption of the active substance. In the case of sunscreens, besides avoiding the transdermal absorption, nanocapsules form a film on the skin surface improving the photoprotection (Alvarez-Román et al. 2001; Jiménez et al. 2004). On the other hand, an increase in the permeation into the deep layers of the skin or high amounts of active substances in the receptor compartment have also been observed using drug-loaded nanocapsules (Miyazaki et al. 2003; Alves et al. 2007; Joo et al. 2008a, b; Kim et al. 2010; Teixeira et al. 2010; Silva et al. 2012). Unfortunately, it is not always possible to compare the results of the penetration/permeation studies due to the lack of standardized methodologies adopted for the authors. Recently the European Commission established a unique definition for nanomaterials; this definition was based on the size of the particles, and in this recommendation nanomaterial is described as: "A natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50 % or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm–100 nm" (OJEU 2011). This definition is relevant to differentiate the formulations by their granulometric profiles based on the number of particles, which was not used, until October 2011, as a characteristic parameter of formulations.

The theoretical mechanisms of penetration and permeation of a nanoencapsulated substance are illustrated in Fig. 13.1. Considering the state of the art, the mechanism of permeation through the skin either of the encapsulated substance or the nanocapsules is still not clear. Different pathways of permeation across the *stratum corneum* have been recognized like the intercellular, transcellular, and appendageal pathways (through hair follicles or sweat glands) (Prow et al. 2011). Although this latter route represents only 0.1 % of the total skin area, it was found that hair follicles play an important role in the permeation of nanoencapsulated substances (Lboutounne et al. 2002). Studies have been carried out to identify if nanocapsules, themselves, are able to penetrate into the skin or if only the drug is released from nanocapsules penetrating by diffusion/dissolution into the skin. This is a controversial issue and should be approached with caution. Some authors reported that nanocapsules can penetrate into the viable skin layers (Weiss-Angeli et al. 2010), and other demonstrated that nanocapsules remain along the *stratum corneum* (Kim et al. 2010). Furthermore, when flexible nanocapsules were evaluated, nanocapsules were found into deep skin layers (Teixeira et al. 2010).

Different preparation methods are described in the literature to produce polymeric nanocapsules such as interfacial polymerization (Al Khouri et al. 1986), interfacial precipitation of preformed polymer (Fessi et al. 1989), emulsification–diffusion (Quintanar-Guerrero et al. 1998), and salting out (Ibrahim et al. 1992). The physicochemical characteristics of the nanocapsules (e.g., particle size, zeta potential, and drug release) are related to the preparation method (Mora-Huertas et al. 2012), and consequently, those properties influence the skin penetration/permeation of the active substances. The interfacial precipitation of preformed polymer is the most common method used to produce nanocapsules intended for drug skin delivery.

13.2 Nanocapsule Production

13.2.1 Nanocapsules Produced by Interfacial Deposition of Polymer

The polymer interfacial deposition technique may also be referred as solvent displacement or nanoprecipitation. Interfacial deposition of polymer is the most used method due to the vesicular architecture of those particles, the

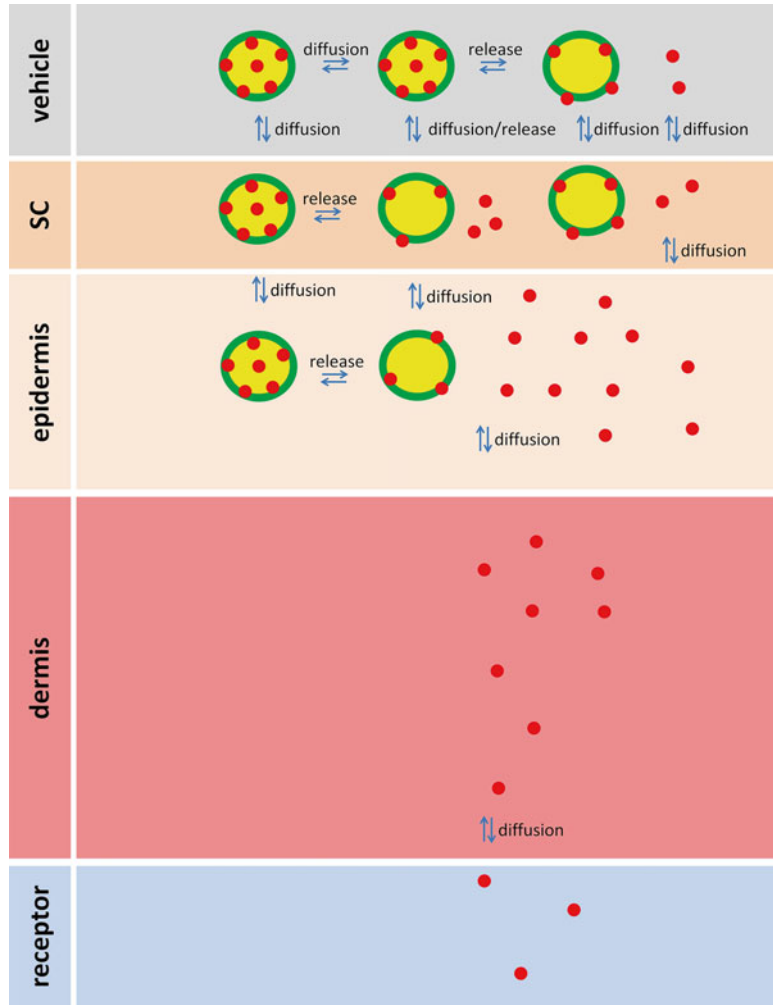

Fig. 13.1 Illustrated penetration and permeation theoretical mechanisms of a nanoencapsulated substance through the vehicle, *stratum corneum* (SC), viable epidermis (epidermis), dermis, and receptor medium

rapid and easy executable technique, as well as the applicability to a large range of raw materials (Schubert et al. 2011). One of its most cited advantages, when compared to other nanocapsule preparation methods, is the low energy required to obtain nanostructures with narrow size distributions (e.g., no high-shearing homogenization, milling, nor sonication) (Hornig et al. 2011). Although it is easy to perform, a careful study of pre-formulation is fundamental to optimize the qualitative and quantitative composition of the product, avoiding the simultaneous formation of nanoemulsions, nanospheres, and microparticles with nanocapsules (Pohlmann et al. 2008; Venturini et al. 2011).

This method requires the use of a water-miscible organic solvent, which forms the organic phase (solvent phase). In this phase, a polymer, an active substance, oil, and a lipophilic surfactant are added to the organic solvent or a mixture of solvents forming a solution (e.g., acetone, ethanol). An aqueous phase is also required and is often called non-solvent phase. The non-solvent phase is composed of water or buffer solution and one or more hydrophilic surfactants especially poloxamer 188 or polysorbate 80 (Mora-Huertas et al. 2010). Nanocapsules prepared by nanoprecipitation at laboratory scale usually present particles with mean diameters between 100 and 400 nm expressed by intensity as a function of hydrodynamic diameters, determined by photon

correlation spectroscopy fitting the autocorrelation function using the cumulant method. The model of those particles corresponds to a polymeric wall adsorbing or not the drug molecules, an oily core dispersing the active substance using a surfactant system commonly composed by two surfactants or a surfactant and an emulsifier. Specifically, when the core of the nanocapsules is composed of a lipid dispersion of an oil and sorbitan monostearate, this nanocarrier has been named as lipid-core nanocapsules (Jäger et al. 2009). In this case, the non-solvent phase is composed by a micellar aqueous solution of polysorbate 80, and the solvent phase is a Newtonian solution of the organic materials. The mechanism of the lipid-core nanocapsule self-assembling to form these colloidal systems was recently proposed (Jornada et al. 2012). The polymeric nanocapsules (lipophilic liquid core) are more flexible than the lipid-core nanocapsules (lipophilic dispersed core) (Fiel et al. 2011). Some representative raw materials used to produce polymeric nanocapsules for cutaneous application are presented in Table 13.1.

The polymers used to formulate nanocapsules are biocompatible and often biodegradable such as polyesters, especially poly(ε-caprolactone) (PCL), poly(D,L-lactide) (PLA), and poly(lactide-co-glycolide) (PLGA). Acrylate polymers are also used like Eudragit® S100, poly(methyl methacrylate) (PMMA), and poly(methacryloyl β-alanine) (PMBA). There is a preference for synthetic polymers due to their purity and inter-batch reproducibility. On the other hand, natural polymers, such as chitosan, have been also used when a specific biological characteristic is required (Bender et al. 2012; Mazzarino et al. 2012). There is a special interest in using cationic polymers when considering formulation intended for topical application, because of the positive charge of the colloidal particles, which can facilitate their interaction with the epithelium, improving the ability to transport the active substances into the skin (Kim et al. 2010; Siqueira et al. 2011).

The oil core of polymeric nanocapsules has a structural or a functional role, acting as a solvent or dispersant for the active substance or being the

Table 13.1 Examples of raw materials used to produce nanocapsules by interfacial deposition of polymer for cutaneous permeation/penetration studies

Polymer	Oil	Active substance	Hydrophilic surfactant	Hydrophobic surfactant	Ref.
PLC	Labrafac hydrophile WL 1219®	Chlorhexidine	Polysorbate 80	Lecithin (Epikuron® 200)	Lboutounne et al. (2002)
PCL	OMC	OMC	Polysorbate 80	–	Jiménez et al. (2004)
PCL	Medium-chain triglycerides	Nimesulide	Polysorbate 80,	Sorbitan monostearate	Alves et al. (2007)
Modified PMMA and PMBA	Squalane	Coenzyme Q10	Sodium lauryl sulfate	–	Kim et al. (2010)
PLA	Retinyl palmitate	Retinyl palmitate	Pluronic® F68, polysorbate 80, Tinogard® Q	Sorbitan monostearate	Teixeira et al. (2010)
Chitosan	Medium-chain triglycerides	Benzophenone-3	Polysorbate 80	Lecithin (Epikuron® 170)	Siqueira et al. (2011)
PCL	Vitamin K	Vitamin K	Polysorbate 80	Sorbitan monostearate	Silva et al. (2012)
Eudragit® S 100	Medium-chain triglycerides	Melatonin	Polysorbate 80	Sorbitan monooleate	Hoffmeister et al. (2012)

PCL poly(ε-caprolactone), *OMC* octyl methoxycinnamate, *PMMA* poly(methyl methacrylate), *PMBA* poly(methacryloyl β-alanine), *PLA* poly(D,L-lactide). Labrafac hydrophile WL 1219®, caprylic/capric triglyceride poly(ethylene glycol)-4 esters; Pluronic, ethylene oxide/propylene oxide block copolymer; Tinogard Q, tris(tetramethylhydroxypiperidinol) citrate; Epikuron® 200, hydrogenated phosphatidyl choline-enriched lecithin; Epikuron® 170, purified phosphatidylcholine; Eudragit S 100, methacrylic acid–methyl methacrylate copolymer (1:2)

active substance itself, such as octyl methoxycinnamate (OMC) (Jiménez et al. 2004), retinyl palmitate (Teixeira et al. 2010), and vitamin K1 (Silva et al. 2012). Mostly, the oil is chosen based on its solvent properties and for this reason different types of triglycerides are widely used (Mora-Huertas et al. 2010). Medium-chain triglycerides are extracted from natural sources, but are highly processed and purified. Synthetic oils have also been explored as core of polymeric nanocapsules in drug permeation evaluations (Kim et al. 2010). In order to improve the drug encapsulation efficiency, additional surfactants may be used, such as sorbitan esters and phospholipids (Alves et al. 2007; Detoni et al. 2012). Vegetable oils such as grape seed oil and almond kernel oil have also been used to obtain polymeric nanocapsules (Almeida et al. 2009). However, permeation studies and assays related to the topical application of these nanocapsules have not been investigated yet.

The continuous phase of the nanocapsule suspensions is usually an aqueous solution. For this reason, the incorporation of these particles into different semisolid formulations is facilitated using carbomers and cellulose-based gel formers (Lboutounne et al. 2002; Alves et al. 2007; Ourique et al. 2010; Weiss-Angeli et al. 2010). Semisolid emulsions (i.e., creams) have been less explored, but also represent a possibility to formulate semisolids containing polymeric nanocapsules (Jiménez et al. 2004). The physicochemical properties of the nanocapsules, the continuous phase and the skin permeation, and/or penetration behavior of polymeric nanocapsules prepared by interfacial deposition of polymer are summarized in Table 13.2.

13.2.2 Nanocapsules Produced by Other Techniques

Besides the polymer interfacial deposition technique, several other methods have been used to produce nanocapsules, such as emulsification–diffusion, salting out (starting from preformed polymer), polymerization in emulsions, and interfacial polymerization (starting from monomers). Table 13.3 summarizes some examples of nanocapsule formulations considering their physicochemical properties, continuous phase and skin permeation, and/or penetration behavior.

13.2.2.1 Emulsification–Diffusion Technique

The emulsification–diffusion technique is widely used to produce nanocapsules (Quintanar-Guerrero et al. 1998; Olvera-Martínez et al. 2005; Calderilla-Fajardo et al. 2006; Joo et al. 2008a). This technique starts with the preparation of an oil/water emulsion under vigorous agitation using a rotor/stator mixer. The internal phase of this emulsion contains an organic solvent partially hydro-miscible and previously saturated with water, the polymer, the oil, and the active substance. The external phase is an aqueous solution containing a surfactant or an emulsifier. Subsequently, additional water is poured into the system leading to the diffusion of the organic solvent to the external phase, and consequently, the polymer is precipitated, an interfacial phenomenon takes place, and the nanocapsules are formed. Each drop of the primary emulsion results in one particle of smaller size (Poletto et al. 2008). Afterward, the organic solvent is removed by evaporation (Quintanar-Guerrero et al. 1998).

Similarly to the interfacial deposition technique, the most used polymers in emulsification–diffusion are polyesters, such as PCL and PLA, and methacrylates, such as Eudragit® series. Among the possibilities of partially hydro-miscible solvents, ethyl acetate is the most common, but propylene carbonate, benzyl alcohol, and dichloromethane are also used. As mentioned earlier, the external phase is composed by water containing a stabilizing agent, such as poly(vinyl alcohol) (PVA), poloxamer, or a surfactant (Mora-Huertas et al. 2010).

This technique has elevated encapsulation efficiency, batch-to-batch reproducibility, easy scale-up, and narrow diameter distribution. On the other hand, there are some inconveniences in using a high volume of aqueous phase that will be evaporated, and this method is not useful for encapsulating hydrophilic drugs due to the water displacement during the diffusion step (Reis et al. 2006).

Table 13.2 Nanoencapsulated substance, particle size and zeta potential, vehicle of the product, model for evaluation, and main results of in vitro skin distribution observed for nanocapsules obtained by interfacial deposition of polymer

Substance	Particle size (nm)/ ζ Potential (mV)[a]	Vehicle (excipient)	Model	In vitro skin distribution	Ref.
Chlorhexidine	278 ± 6/ +32.4 ± 0.1	Hydrogel (carboxymethylcellulose)	In vitro, tape stripping, SEM, pig ear skin	Hydrogels containing NC enhanced the drug absorbed into the SC. Nanocapsules were visualized in SC surface and in skin follicles	Lboutounne et al. (2002)
OMC	255 ± 5/ND	NC suspensions	In vitro, vertical diffusion cell, pig ear skin	OMC was not detectable in the receiver compartment. Higher amounts were found in the SC	Alvarez-Román et al. (2004)
OMC	ND	O/W and W/O emulsions	In vitro, static Franz diffusion cell, flank pig skin	NC decreased the penetration of OMC in the SC. Both vehicles containing NC decrease the skin penetration of OMC	Jiménez et al. (2004)
Chlorhexidine	278 ± 6/+32.4 ± 0.1	NC suspensions	In vitro, static Franz diffusion cells, full-thickness and stripped hairless rat skin	Drug permeability was higher through stripped as compared to full-thickness skin. The drug release from the nanocapsules influenced the permeation of chlorhexidine	Lboutounne et al. (2004)
Nimesulide	277/ND	Hydrogel (Carbopol 940®)	In vitro, Franz-type diffusion cells, abdominal human skin	Hydrogels containing nanocapsules promoted greater drug penetration in the skin	Alves et al. (2007)
CoQ_{10}	ND	NC suspensions	In vitro, Franz-type vertical diffusion cell system, abdominal skin of Hartley albino guinea pigs	NC with positive-charge surface increased the penetration of CoQ_{10}	Kim et al. (2010)
Retinyl palmitate	215/−14.9 ± 5	Hydrogel (Carbopol 940®)	In vitro, Franz-type diffusion cells, abdominal human skin	Higher amounts of retinyl palmitate were found in the viable skin layers and the drug was also detected in the receptor chamber	Teixeira et al. (2010)
OMC	263 ± 2/ND	Hydrogel (HEC)	In vitro, Franz-type diffusion cells, pig flank skin	Nanoencapsulation accumulated in skin surface and limited release of OMC in the deeper skin layers	Weiss-Angeli et al. (2010)

Tretinoin	213 ± 6/−6.8	NC suspension and hydrogel (Carbopol® Ultrez NF 10)	In vitro, static Franz diffusion cells, human heat-separated epidermis	Tretinoin incorporated in NC permeates less through the skin, remaining for more time on the skin surface. The hydrogel did not affect the skin permeation profile of nanoencapsulated tretinoin	Ourique et al. (2011)
Benzophenone-3	Uncoated NC: 175 ± 1/−8 ± 1 Coated NC: 202 ± 7/+21 ± 7	Hydrogel (HEC)	In vitro, Franz-type diffusion cells, abdominal pig skin	The cationic coating was more efficient in maintaining the active substance on the SC with reduced permeation through the skin	Siqueira et al. (2011)
Isotretinoin	231 ± 9/−9.1 ± 1.9	Hydrogel (HEC)	In vitro, Franz-type diffusion cells, human and pig abdominal skin.	Nanoencapsulation increased isotretinoin penetration in the SC without permeation through the skin. A positive correlation coefficient between human and pig skin penetration were observed	Bettoni et al. (2012)
Resveratrol	266 ± 0.72/ND	NC suspensions	In vitro, Franz-type diffusion cells (protected from light and under UVA radiation), pig skin	In the dark, large amounts of resveratrol were found in the dermis. Under UVA radiation, nanoencapsulation promoted the retention of the resveratrol on the SC and in the skin surface	Detoni et al. (2012)
Melatonin	NC suspension: 180 ± 24/−11.2 ± 5 Dried NC using lactose: 192 ± 50/ND Dried NC using maltodextrin: 181 ± 27	Hydrogel (Carbopol 940®)	In vitro, Franz-type diffusion cells, pig abdominal skin	Lower melatonin permeated through the skin for the hydrogels prepared with spray-dried nanocapsules. This result corroborates with drug release studies	Hoffmeister et al. (2012)
Vitamin K1	211 ± 2/−14.9 ± 0.6	NC suspensions	In vitro, Franz-type diffusion cells, female pig skin	Nanoencapsulation retained the active in the skin layers without permeation through the skin	Silva et al. (2012)

SEM scanning electronic microscopy, *NC* nanocapsule, *SC stratum corneum*, *OMC* octyl methoxycinnamate, *ND* not determined, *O/W* oil in water, *W/O* water in oil, *CoQ_{10}* coenzyme Q_{10}, *HEC* hydroxyethylcellulose

[a]The measurements were performed from nanocapsule suspensions

Table 13.3 Nanoencapsulated substance, particle size, vehicle of the product, model for evaluation, and main results of in vitro skin distribution observed for nanocapsules obtained by other methods

Substance	Method of preparation	Particle size (nm)[a]	Vehicle	Model	In vitro skin distribution	Ref.
Indomethacin	Interfacial polymerization	188	NC formulation dispersed in pH 7.4 phosphate buffer or PLF-127 gel	In vitro, plastic diffusion cell, rat skin	The flux and the cumulative amount which permeated the indomethacin of formulation NC in pH 7.4 phosphate buffer were greater than the NC in PLF-127 gel or only PLF-127 gel	Miyazaki et al. (2003)
OMC	Emulsification–diffusion	458.3	NC suspensions	In vivo, tape stripping, human skin	Total amount of OMC penetrated in the SC was lower for NC formulation than for NE	Olvera-Martínez et al. (2005)
OMC	Emulsification–diffusion	458.3 NC containing sucrose laurate: 417.3 NC containing oleate sucrose: 362.8	NC suspensions containing sucrose laurate or sucrose oleate	In vivo, tape stripping, human skin	The OMC penetration was influenced by formulation type and the presence of sucrose esters. Total penetrated amount of OMC was lower for NC than for NE and conventional emulsion. The laurate sucrose in nanoemulsion increases the OMC penetration	Calderilla-Fajardo et al. (2006)
Hinokitiol	Emulsification–diffusion	356	NC suspensions	In vitro, Franz diffusion cell, female hairless mice skin	The hinokitiol permeation across the hairless mice skin was higher for NC than for solutions	Joo et al. (2008a)
Hinokitiol	Emulsification–diffusion	NCs prepared using CTAC: 223 SLS: 361 CTAC/gelatin: 598	NC suspensions	In vitro, Franz diffusion cell, female hairless mice skin	The flux of hinokitiol through hairless mice skin was higher for NC prepared using SLS than NC prepared using CTAC or CTAC/gelatin	Joo et al. (2008b)
OMC	Salting-out technique	$d_{50\%} < 670$ and $d_{90\%} < 1070$	NC contained in an O/W emulsion gel	In vitro, Franz diffusion cell, pig skin	The OMC nanoencapsulated was retained at the skin surface. The OMC release from NC in viable skin layers was only 25 % and ~40 % after 1 and 2 or 3 h of that quantified after skin penetration of free OMC	Vettor et al. (2010)
OMC and avobenzone	Spontaneous emulsification combined to interfacial polycondensation	OMC NC: 282 Avobenzone NC: 339 OMC/avobenzone NC: 446 OMC/α-toc NC: 311 Avobenzone/α-toc NC: 385 α-toc NC: 270	NC suspensions	In vitro, Franz diffusion cell, pig skin	All formulations showed low epidermis penetration. There was no difference between NC and NE	Hanno et al. (2012)

NC nanocapsule, *PLF-127* Pluronic F-127, *OMC* octyl methoxycinnamate, *NE* nanoemulsion, *CTAC* cetyltrimethylammonium chloride, *SLS* sodium lauryl sulfate, *α-toc* α-tocopherol

[a]The measurements were performed for nanocapsule suspensions

13.2.2.2 Salting Out

The salting out is also a technique to obtain nanocapsules by using preformed polymers. In this method the goal is to separate a hydro-miscible solvent from water using the salting-out effect. The technique has two steps; the first step consists in emulsifying an organic phase containing an organic solvent, a polymer, an oil, and the active substance with an aqueous phase containing a colloidal stabilizer, such as poly(vinyl pyrrolidone) (PVP) or hydroxyethylcellulose (HEC) and a salting-out agent. On the second step, an excess of water is added inducing the diffusion of the organic solvent and the formation of the nanocapsules. The salting-out agent is usually an electrolyte, such as magnesium chlorate, magnesium acetate, or calcium chlorate, or a nonelectrolyte, such as sucrose. At the end of the process, the organic solvent and the salting-out agent are removed by ultrafiltration or ultracentrifugation (Ibrahim et al. 1992; Reis et al. 2006). Salting-out technique does not require a high temperature being useful to encapsulate thermosensitive substances. Some drawbacks are the exclusive use of lipophilic substances and the need of an additional purification step to remove the salting-out agent (Reis et al. 2006).

13.2.2.3 Interfacial Polymerization and Interfacial Polycondensation

The interfacial polymerization technique produces nanocapsules starting from monomers. In general, the monomer is dissolved in an organic solvent (acetone, ethanol, *n*-butanol, among others) containing the active substance and oil. This organic solution is injected into an aqueous solution of a surfactant or an emulsifier under agitation. When in contact with water, the oil forms a colloidal dispersion; the organic solvent diffuses to the aqueous phase and the polymerization initiates at the monomer/water interface. The method is widely used to produce particles containing poly(alkyl cyanoacrylates), poly(phenyl esters), and poly(urethanes). In the case of poly(alkyl cyanoacrylates), an advantage is the rapid polymerization, whose mechanism is based on an anionic polymerization and whose stability of particles is dependent of the molar mass of the chains (Al Khouri et al. 1986; Reis et al. 2006). Among the advantages of this method, we can cite the high encapsulation efficiency and that the polymerization in situ facilitates the covering of the oil droplet contours. The main disadvantage is the need of a purification step to separate the unreacted monomers.

Nanocapsules are also prepared by spontaneous emulsification combined to an interfacial polycondensation reaction. In this technique, the nanocapsules are formed by the polycondensation of two monomers, a lipophilic and a hydrophilic. Hanno and coworkers (2012) prepared polyamide nanocapsules using sebacoyl chloride and diethylenetriamine added in the organic phase and aqueous phase, respectively (Hanno et al. 2012).

13.3 Parameters Influencing Skin Penetration/Permeation

13.3.1 Polymeric Wall

The polymeric wall can influence the release, penetration, and permeation of the active substances loaded in the nanocapsules by acting as a diffusion barrier. In this aspect, variables such as wall density, thickness, homogeneity, and porosity are important, as well as chemical constituents and their functionality. In this section we selected the penetration/permeation studies based on the influence of the polymeric wall on the drug behavior.

Nanocapsules modify the release profiles of nanoencapsulated active substances and, as a consequence, alter their cutaneous penetration or permeation (Contri et al. 2010). Furthermore, nanocapsules modify the drug flow through biological membranes, such as the skin (Ourique et al. 2011). The comparative study of drug permeation after applying nanocapsules or nanoemulsion is particularly interesting to define the influence of the polymeric wall, as a barrier, and to determine the interactions of the nanocarriers with the different layers of the skin.

Nanoemulsions are nanotechnological formulations formed of dispersed oil droplets in

aqueous medium, stabilized by a surfactant system. They are more flexible systems than polymeric nanocapsules. This characteristic may differentiate their penetration profile in the skin. Studies have compared nanocapsules to their analogous nanoemulsions in order to establish the influence of the polymer barrier. In addition, by comparing nanocapsules to an analogous nanospheres composed without the oil, it was possible to determine whether the polymeric material or the supramolecular architecture is most important for the drug permeation profile (Alves et al. 2007).

In general, the drug release behavior can offer information, which can be related to the ability of the drug to cross biological barriers. Even though the polymeric wall is capable of reducing the release rate of substances, such as clobetasol propionate (Fontana et al. 2011) when compared to a nanoemulsion, aspects beyond the release behavior have to be pondered considering the skin as a membrane. The polymeric wall interacts with the horny layer, which acts as a reservoir for the topically applied substances. Alves and coworkers (2007) found that nimesulide-loaded nanocapsules provided a larger drug penetration into the *stratum corneum* and deeper skin than the drug-loaded nanoemulsion. When nimesulide-loaded nanocapsules were compared to nimesulide-loaded nanospheres, the authors confirmed that the polymer (PCL) was the material responsible for the interaction of the nanocarriers with the horny layer, since the amount of nimesulide in the *stratum corneum* was similar for both formulations. On the other hand, the amount of drug in the viable layers of the skin was larger for the nanocapsules than for the nanospheres, indicating that the supramolecular architecture defines the drug penetration into the deeper layers of the skin (Alves et al. 2007).

The clinical trial of cutaneous penetration carried out with OMC-loaded nanocapsules prepared by emulsification–diffusion (458 nm), OMC-loaded nanoemulsion (161.7 nm), and a conventional emulsion containing OMC (2814 nm) (Olvera-Martínez et al. 2005). A higher penetration of OMC from nanoemulsion was observed. Calderilla-Fajardo and coworkers (2006) corroborate those results studying the similar nanocapsules and nanoemulsions (Calderilla-Fajardo et al. 2006). The greater penetration of the OMC-loaded nanoemulsion is probably due to their particle sizes and high deformability. On the other hand, the low penetration of OMC in the *stratum corneum* after the administration of OMC-loaded nanocapsules may be related to their larger diameter when compared to the nanoemulsion formulation. Moreover, the rigidity of the nanocapsules compared to the nanoemulsion, due to the presence of cellulose acetate phthalate as polymer wall, restrained the OMC passage through the skin (Olvera-Martínez et al. 2005; Calderilla-Fajardo et al. 2006).

Two independent studies (Vettor et al. 2010; Weiss-Angeli et al. 2010) evaluated the permeation of OMC-loaded nanocapsules using pig skin, as membrane. Two extraction techniques were proposed: one to determine the total OMC content in the skin by extracting and recovering it and the other to determine only the OMC released from the nanocapsules in the skin using a solvent for OMC that presents low diffusion to the core of nanocapsules, avoiding its extraction from the nanocapsules and recovering exclusively the OMC released in the skin. The first study demonstrated that OMC was released from the PLA nanocapsules on the skin surface, in the *stratum corneum*, epidermis, and dermis by comparing the OMC amounts using both extraction techniques. For PCL nanocapsules, similar results were observed considering the *stratum corneum* and epidermis; however, no statistically significant difference was observed between both extraction techniques in the dermis. In this case, only OMC reached the dermis, while for PLC nanocapsules the particles also reached this layer. No OMC was detected in the receptor fluid independent of the polymer used as wall. Furthermore, 80 % of the dose applied on the skin was recovered at the surface, indicating infinite dose conditions. The use of different materials (PCL and PLA) as polymeric wall of OMC-loaded nanocapsules led to different results. Interestingly, the release of OMC from PLA nanocapsules or PCL nanocapsules was faster on the skin surface and

superficial layers than in the deeper layers of the skin. The authors attribute the fast release at the surface and superficial layers of the skin to the environment that, differently from the other layers, can allow the degradation of the polymeric wall (Vettor et al. 2010) or permit the formulation to dry leading to a destabilization of the nanocapsules. Another hypothesis is that the upper layers of the skin are more lipophilic (10–13 % of water (w/w)) facilitating the release of OMC, while the deeper layers have a higher content of water (almost 70 %, w/w) (Weiss-Angeli et al. 2010).

Similarly, a previous study performed by Hanno and coworkers (2012) evaluated the epidermal penetration into pig skin of two different sunscreens in polyamide nanocapsules obtained by spontaneous emulsification combined to interfacial polycondensation. The influence of the polymeric wall was first assayed using a membrane-free model. For both sunscreens, the release from the nanoemulsions after 24 h was about 3 times higher than their release from the polyamide nanocapsules. The polyamide wall was capable of controlling the release of the sunscreens. All formulations (nanocapsules and nanoemulsions) provided very low epidermal penetration of the actives. Since no differences were observed in the penetration of sunscreens from nanoemulsions and nanocapsules, the influence of the polymeric wall was not confirmed. The low epidermal penetration is probably due the high affinity of the sunscreens with the *stratum corneum* (Hanno et al. 2012).

13.3.2 Surface Functionalization

The zeta potential reflects the surface potential of particles and can be influenced by changes on the interface of the nanosystem induced by the dispersant medium; such changes are dependent on the chemical nature of the polymer and related to the stabilizing agent and/or the pH of the bulk solution (Mora-Huertas et al. 2010). The zeta potential values can be negative or positive and are usually determined by electrophoretic light scattering (Schaffazick et al. 2003; Mora-Huertas et al. 2010). In modulus, an elevated zeta potential value is important to guarantee the physical stability of colloidal dispersions due to the repulsive forces that avoid collisions between neighboring nanoparticles. The relevance of this parameter to the physicochemical stability of colloids is higher for the electrostatic stabilization mechanism than for the steric stabilization. Besides influencing the physicochemical stability of the formulations, the particle surface may also influence the behavior of the nanocapsules at the skin surface.

Nanocapsules with a negative zeta potential, mainly constituted of polyesters [poly(ε-caprolactone) and poly(D,L-lactide)] and coated with non-ionic surfactants (polysorbate 80 and poloxamers), are generally proposed to control the penetration/permeation of active substances in the skin (Mora-Huertas et al. 2010; Teixeira et al. 2010; Ourique et al. 2011; Hoffmeister et al. 2012; Silva et al. 2012). In order to improve the electrostatic interaction between nanocarriers and the negatively charged biological surfaces, nanoparticles have been prepared with materials that promote positive surface charge (Contri et al. 2010; Kim et al. 2010; Siqueira et al. 2011). Chitosan, a cationic polymer, is the most common material used for this purpose. The excellent properties like biodegradability, biocompatibility, bioadhesion, and antibacterial activity make this polysaccharide widely used in drug delivery systems (Calvo et al. 1997; Contri et al. 2010; Siqueira et al. 2011; Mazzarino et al. 2012; Ridolfi et al. 2012).

The effect of cationic polymeric nanocapsules on the in vitro skin penetration/permeation of a sunscreen was recently demonstrated (Siqueira et al. 2011). Benzophenone-3-loaded chitosan-coated and benzophenone-3-loaded uncoated PCL nanocapsules were prepared, and the in vitro skin penetration was evaluated using abdominal pig skin, as membrane in static Franz diffusion cells. HEC hydrogels containing benzophenone-3-loaded chitosan-coated or unloaded and/or uncoated nanocapsules were prepared to evaluate the influence of the positive surface charge of the nanocapsules on the benzophenone-3 skin penetration profile. Zeta potential analyses indicated that the particles were effectively coated since the

values were −8 ± 1 mV for the uncoated nanocapsules and +21 ± 1 mV for the chitosan-coated nanocapsules. Cationic nanocapsules were able to retain the sunscreen in the *stratum corneum* for a longer period and to reduce its concentration in the receptor compartment. Those finding suggest that the formulation can decrease the sunscreen transdermal transport, consequently enhancing the sunscreen efficacy and avoiding or limiting the toxicological problems caused by benzophenone-3 absorption. The higher retention of nanoencapsulated benzophenone-3 using cationic particles in the superficial layers of the skin was explained by three distinct mechanisms: (1) the bioadhesive properties of the polymer (chitosan), (2) the electrostatic interaction between chitosan and the epithelium, and (3) the close contact between the nanocapsules and the skin (Siqueira et al. 2011).

The effects of the positive surface charge were also observed for chlorhexidine base-loaded PCL nanocapsules by Lboutounne and coworkers (2002). Unloaded PCL nanocapsules showed negative zeta potential; however, drug-loaded nanocapsules had positive zeta potential. The authors suggested that chlorhexidine ($pK_{a1} = 10.3$ and $pK_{a2} = 2.2$) is adsorbed as a dictation specie to the nanocapsule-solvent interface (pH 4–8) giving a positive zeta potential to the formulation. An increase in the accumulation of nanoencapsulated chlorhexidine in the *stratum corneum*, enabling a prolonged topical antimicrobial activity, was demonstrated with a tape stripping assay. This increase may be related, among other reasons, to the strong bioadhesion of the carrier to the skin surface, allowing the controlled release of the drug. Furthermore, based on scanning electron microscopy analysis, the deposition of nanoparticles on the surface of the skin and a follicular transport were also suggested (Lboutounne et al. 2002).

Unlike the previous results, the skin penetration of coenzyme Q10 increased when administered in cationic nanocapsules composed of a copolymer, as wall, and squalane, as oil core. According to the authors, this increase is due to the deformability property of the nanocapsules and to the interaction between the carrier and the intercellular lipid lamellae of the *stratum corneum* (Kim et al. 2010).

As mentioned earlier, the pH of the bulk solution can influence the zeta potential and, consequently, the physical stability of the nanosystems (Mora-Huertas et al. 2010). Increasing the pH from 2 to 10, PCL nanocapsules prepared by emulsification–diffusion using sodium dodecyl sulfate as stabilizer showed an increase of the absolute value of the zeta potential reaching values of −30 mV. The mean diameter of the particles was not altered indicating that the electrostatic repulsion maintained the colloidal physical stability. On the other hand, nanocapsules containing cetyltrimethylammonium chloride as stabilizer showed a zeta potential of +20 mV at pH 2, decreasing to zero at pH 10. Consequently, the mean diameter of the particles varied from 200 nm to approximately 14 μm due to the aggregation (Joo et al. 2008b). Aggregated particles may have distinct skin permeation profiles as compared to the administration of their primary particles.

The cutaneous permeation of hinokitiol-loaded nanocapsules was evaluated at a physiological pH value. After 20 h of experiment, the monoterpenoid permeated more through hairless mice skin, when the sodium dodecyl sulfate nanocapsules were administered, compared to the administration of cetyltrimethylammonium hinokitiol-loaded nanocapsules. Anionic surfactants can be used as absorption enhancers, and sodium dodecyl sulfate might have acted enhancing the permeation of hinokitiol from the nanocapsules. Negatively charged nanocapsules, containing rhodamine, were evaluated by confocal microscopy. The fluorescent dye was not detected in the *stratum corneum*, epidermis, or dermis, suggesting that the nanocapsules did not penetrate into hairless mice skin. The larger amount of hinokitiol which permeated into the skin was likely due to the nanocapsule deposition on the skin surface instead of the nanocapsule skin permeation (Joo et al. 2008b). The improvement of the cutaneous permeation of this monoterpenoid by using anionic-coated nanocapsule was compared to the hinokitiol solution. The flow of the nanoencapsulated hinokitiol permeation in

hairless mice skin was higher than that observed for the hinokitiol solution in propylene glycol (Joo et al. 2008a).

13.3.3 Vehicle

The conversion of polymeric nanocapsules into semisolid formulations has the advantage of easier application of the product. Polymeric nanocapsules are usually incorporated into hydrogels (e.g., HEC and carbomers) (Fontana et al. 2011; Siqueira et al. 2011; Hoffmeister et al. 2012) and into emulsions (Jiménez et al. 2004; Vettor et al. 2010) as previously commented. To prepare hydrogels as final products, the water is partially or completely substituted by the nanocapsule suspension (Weiss-Angeli et al. 2010; Ourique et al. 2011). On the other hand, to prepare emulsions such as creams or lotions, the nanocapsule suspension can be incorporated to a cream previously prepared (Jiménez et al. 2004), or it can be added as the phase aqueous during the emulsification if the dispersant (e.g., *iso*propyl myristate) is a non-solvent for the colloid and the subsequent steps are performed at room temperature (Vettor et al. 2010).

The main pathways for the delivery of an active substance, loaded in polymeric nanocapsules, to the skin layers occur in different steps (Fig. 13.1). The drug/carrier, the drug/vehicle, and the drug/skin partitions define the physicochemical behavior of formulations, as well as the flexibility and (physical or chemical) solubility of the nanocarriers. Different studies of drug skin penetration and permeation after topical application of polymeric nanocapsules have been developed in the past 10 years.

Ourique and coworkers (2011) compared the flux, lag time, and permeability coefficient of tretinoin-loaded nanocapsules using human heat-separated epidermis. There were no statistically significant differences in these permeation parameters when the tretinoin-loaded nanocapsule suspension was compared to the tretinoin-loaded nanocapsule carbomer gel (Carbopol® Ultrez NF 10). The similar behavior of those formulations may be attributed to the tretinoin lipophilicity (log $P = 5.66$). Since the drug is released from the nanocapsules, similar water/*stratum corneum* and hydrogel/*stratum corneum* partitions are expected. Furthermore, the drug flux and permeability were lower, and the respective lag times were higher for the tretinoin-loaded nanocapsules compared to the nonencapsulated drug either in suspension or in polyacrylic acid gel, i.e., carbomer gel (Carbopol® Ultrez NF 10). This result confirmed that the release control of the drug permeation into the skin from the tretinoin-loaded nanocapsules is dependent of the tretinoin release from the nanocapsules to the vehicle (Ourique et al. 2011).

Different molecular substances can be used as skin penetration enhancers of drugs. The simultaneous use of penetration enhancers and polymeric nanocapsules was investigated. The cutaneous penetration of OMC-loaded nanocapsule and nanoemulsion suspensions containing sucrose laurate or sucrose oleate, as penetration enhancer, was evaluated in volunteers (Calderilla-Fajardo et al. 2006). The effects of the absorption promoters were studied by comparing nanocapsules and nanoemulsions containing or not penetration enhancers. It was observed that sucrose laurate in nanoemulsions significantly improved the penetration of OMC into the *stratum corneum*, but the penetration was apparently higher for OMC-loaded nanocapsules when compared to the formulation without penetration enhancers. The skin treated with formulations containing sucrose oleate did not modify the penetration profile of the sunscreen.

In 2004, Jimenéz and coworkers sought out the difference between the permeation of four formulations: OMC loaded in nanocapsules or free in oil/water and water/oil emulsions. No variation was observed in the transdermal permeation of OMC when using these formulations. The formulations containing OMC-loaded nanocapsules did not provide difference in permeation when compared to the conventional OMC emulsions, but provided higher retention in the upper layers of the skin (Jiménez et al. 2004). The similar permeation and penetration profiles of OMC from both types of emulsions do not clarify the role of the vehicle (oil/water or water/oil emul-

sion or cream) in the penetration of OMC, but indicate that the phase inversion of an emulsion may have only a small influence to the penetration and permeation profile of nanoencapsulated lipophilic ingredients.

Another study with OMC-loaded nanocapsules incorporated in a HEC hydrogel, using pig skin, as a membrane and a Franz-type diffusion cell was also performed (Weiss-Angeli et al. 2010). The experimental protocol is comparable to the previous study described above (Jiménez et al. 2004). The skin retention of OMC, when using semisolid formulations with OMC-loaded nanocapsules, was similar among the emulsions (O/W and W/O) and the hydrogel. Eighty percent of the applied OMC was found in the skin surface and horny layer for both studies. However, for Jimenéz and coworkers (2004) only 8.5 % of this amount was quantified in the *stratum corneum*, whereas for Weiss-Angeli and coworkers (2010), this value was closed to 22 %, when the OMC-loaded nanocapsules in hydrogels were studied. In addition, when OMC was nanoencapsulated and the nanocapsules were incorporated into emulsions (i.e., creams), the semisolid formulation led to a reduction of OMC penetration, while when OMC was nanoencapsulated and the nanocapsules incorporated into a HEC hydrogel, the semisolid formulation led to a higher accumulation of OMC in the horny layers (Jiménez et al. 2004; Weiss-Angeli et al. 2010). The results indicate that lipophilic drugs tend to interact less with hydrophilic vehicles than with creams, favoring the substance accumulation in the *stratum corneum* likely within the nanocapsules as reservoirs.

With a different approach, Lboutounne and coworkers (2004) performed two experiments: an in vitro drug release and a skin permeation study. By overlaying the release and the permeation profiles of neutral chlorhexidine-loaded nanocapsules, the authors observed that a phase of rapid release (0–5 h) is overlapped with the phase of fast permeation (also 0–5 h) and the plateau in the release profile coincides with the slow permeation phase. This comparison allowed the authors to suggest that the permeation of chlorhexidine from nanocapsules through the skin could be controlled by the drug release from the carrier. In this study, the authors hypothesize about the critical step in the sustained permeation without comparing two vehicles (using only water as a vehicle). One of the matters that need to be pondered, as brought up by the authors, is that the properties of the skin as a barrier are being overlooked since the comparison is performed with two different assays, whereas no assay with different vehicles was carried out. By comparing full-thickness skin and tape-stripped skin, the authors were able to confirm that the interaction of the particles with the horny layer influenced the permeation, since chlorhexidine permeation was higher for tape-stripped skin (Lboutounne et al. 2004).

Assays that use skin as a membrane (diffusion barrier) are better correlated to the expected in vivo penetration/permeation. However, preliminary release studies help to define the sustained release system enabling answering questions such as the influence of vehicles. In vitro release studies show that the diffusion through the vehicle and the drug partitioning vehicle/skin are also important steps in the permeation of a formulation containing nanocapsules. A comparative study of the in vitro release of melatonin from drug-loaded nanocapsule suspension and from drug-loaded nanocapsule carbomer hydrogel (Carbopol® 940) revealed that the hydrogel caused a decrease of the melatonin release (Hoffmeister et al. 2012). A similar decrease was also observed comparing nonencapsulated melatonin solution and hydrogel. The results showed that the drug release from the nanocapsules to the vehicle represents a limiting step and probably influences the penetration and permeation of melatonin. Besides, the diffusion of melatonin from different vehicles (suspension and hydrogel) is influenced by their viscosity and rheological behaviors. In this way, it can be noticed that a polar substance, such as melatonin, shows higher interaction with the aqueous media, and thus, the vehicle exerts a higher influence on the drug release, and on the skin penetration, as compared to tretinoin (Ourique et al. 2011), whose hydrophobicity leads to a slower diffusion from the nanocapsule to the vehicle.

In another study, the release profiles of capsaicin-loaded nanocapsules and dihydrocapsaicin-loaded nanocapsules were evaluated (Contri et al. 2010). Different profiles were observed when the release of the capsaicinoids from the nanocapsule suspension was compared to their release from a chitosan hydrogel containing the nanocapsules. Capsaicin showed a slower release from the semisolid vehicle than from the suspension, demonstrating that the limiting step of sustained release is the release from the vehicles. Additionally, capsaicin-loaded nanocapsules presented a slower release profile compared to the dialysis of the nonencapsulated substance indicating that the release from the nanocapsule also represents a limiting step to the release of capsaicin. Moreover, the different dialysis profiles for dihydrocapsaicin were observed when solution and drug-loaded nanocapsules were compared. On the other hand, similar release profiles were observed for dihydrocapsaicin when the nanocapsule suspension was compared to the chitosan hydrogel containing the drug-loaded nanocapsules. Dihydrocapsaicin is a substance presenting higher log P than capsaicin. In this case the release from the nanocapsule is the only critical step for the drug release. Indeed, there is a relation between the Log P of the active substance and the role of the nanocapsule and the vehicle to control the drug release or control the skin penetration of the drug (Table 13.4).

Nanocapsules of poly(*n*-butyl cyanoacrylate) (PNBA) containing indomethacin were prepared by the interfacial polymerization technique (Miyazaki et al. 2003). The cutaneous permeation was studied to evaluate the influence of the vehicle and the presence of the nanocapsules on indomethacin permeation through rat skin. In this way, three formulations were prepared: indomethacin-loaded nanocapsules dispersed in thermosensitive gel obtained with the ethylene oxide/propylene oxide block copolymer (Pluronic® F-127), nonencapsulated indomethacin in Pluronic® F-127 gel, and indomethacin-loaded nanocapsules dispersed in pH 7.4 phosphate buffer. The flow and cumulative amount of indomethacin were the highest for the formulation of nanocapsules dispersed in phosphate buffer. These parameters were lower for the nanocapsules in the thermosensitive gel due to the viscosity of the vehicle, confirming that the vehicle influences the permeation through the skin. The permeation of nonencapsulated and nanoencapsulated indomethacin in the gel was compared and the flow and cumulative amount of indomethacin were lower for the nonencapsulated substance, indicating that the permeation is also related to the presence of the nanocapsules. To confirm that indomethacin topically applied permeates the skin and is absorbed, an in vivo assay was performed with rats. The area under the curve for the nanoencapsulated indomethacin was 6.76 μg h mL^{-1} and for its nonencapsulated form it was 2.04 μg h mL^{-1}.

In addition, to verify if the quantity of indomethacin permeating the skin was a result of the drug release from the nanocapsules or if the nanocapsules containing indomethacin were permeating the skin themselves, a release assay was performed. The drug release profiles from nanocapsules in the phosphate buffer and gel containing indomethacin nonencapsulated were evaluated using cellulose membranes. Twelve percent of indomethacin was released from the nanocapsules in 8 h, a quantity much lower than the obtained in the receptor compartment of the permeation assay with rat skin, indicating that the permeated quantity is a sum of the indomethacin

Table 13.4 Nanoencapsulated drugs, their logarithm of the octanol–water partition coefficient (Log *P*), and the release limiting step to control the drug release or skin penetration

Active substance	Log *P*	Release limiting step	Ref.
Tretinoin	5.66	Release from the NC	Ourique et al. (2011)
Dihydrocapsaicin	4.11	Release from the NC	Contri et al. (2010)
Capsaicin	3.33	Release from the NC + release from the vehicle	Contri et al. (2010)
Melatonin	1.15	Release from the vehicle	Hoffmeister et al. (2012)

NC nanocapsule

released and indomethacin in intact nanocapsules (Miyazaki et al. 2003). It is worth noticing that the release assay using a cellulose membrane must be carefully employed since the pore membranes are irregular having a large size distribution by which nanocarriers could permeate.

Besides the two semisolid pharmaceutical forms widely used, creams and gels, other forms can be used for cutaneous administration, such as emulgels. Vettor and coworkers (2010) developed an emulgel containing OMC or OMC-loaded nanocapsules. The nanoencapsulated substance was better retained on the surface of the skin when compared to the soluble molecule in the emulgel, which is the aim of using sunscreen-loaded nanocapsules. The quantity of OMC in the *stratum corneum* was higher after applying the nanocapsule formulation onto the skin surface than upon the application of the conventional formulation. In agreement with other authors, the quantity of OMC in the horny layer was maintained for 3 h when OMC-loaded nanocapsules were applied, while OMC penetration in the viable layers of the skin was higher for the OMC-loaded emulgel without nanocapsules. The nanoencapsulation is able to retain the active substance in the upper layers of the skin without increasing the transdermal transport (Vettor et al. 2010).

13.4 Therapeutic and Cosmetic Applications

Polymeric nanocapsules have been proposed as drug carriers for topical applications aiming a controlled release and controlled penetration/permeation in the skin. They are also able to increase the drug stability against sunlight and reduce the side effects of drugs and cosmetics (e.g., skin irritation and allergy). Table 13.5 summarizes the main applications and benefits of nanocapsules intended for topical use. As can be seen, the results are promising.

Photoprotectors are topical formulations to be used daily in order to prevent sun damage, such as erythema, photoaging, and skin cancers. The effectiveness of those products is directly related to their affinity for the *stratum corneum*, which enhances the residence time of the active on the skin surface. In this context, nanocapsules have been employed to increase the retention of different chemical sunscreens in the outermost skin layers and improve their effectiveness (Alvarez-Román et al. 2004; Jiménez et al. 2004; Siqueira et al. 2011).

After nanoencapsulation, an increase in the photoprotective property of OMC was observed. Guinea pigs were exposed to UVB radiation, and

Table 13.5 Nanoencapsulated substance, application, and benefits of the nanocapsules for topical use

Substance	Application	Advantages	Ref.
OMC	Photoprotection	Significantly better protection against in vivo UVB-induced erythema	Alvarez-Román et al. (2001)
Chlorhexidine	Antiseptic	Sustained antibacterial effect against the resident and transient hand flora	Nhung et al. (2007)
Hinokitiol	Hair growth promoter	Nanocapsules promoted an increase in hair growth	Hwang and Kim (2008)
OMC + quercetin	Photoprotection	Protection of the active substance from UV radiation	Weiss-Angeli et al. (2008)
Benzophenone-3	Photoprotection	Increased photostability and effectiveness. Nanocapsules do not produce allergenicity in mouse	Paese et al. (2009)
Clobetasol propionate	Anti-inflammatory	Improves in vivo dermatological efficacy for contact dermatitis treatment	Fontana et al. (2011)
Tretinoin	Anti-acne	Improved its photostability	Ourique et al. (2011)
OMC + avobenzone	Photoprotection	Increase in 50 % of the photostability	Hanno et al. (2012)
Resveratrol	Antioxidant	Delayed photodegradation	Detoni et al. (2012)

OMC octyl methoxycinnamate

the nanocapsules improved the capacity of the sunscreen to inhibit UV-induced erythema compared to the control gel containing the nonencapsulated sunscreen. This result indicated that the nanoencapsulated sunscreen adheres to the skin surface, forming a protective film (Alvarez-Román et al. 2001). An increase in the effectiveness of benzophenone-3 has also been observed due to a synergic effect between the chemical sunscreen and the presence of the nanocapsules (Paese et al. 2009).

The sunscreen efficacy is not only related to the capacity of adhesion of the product on the *stratum corneum* but also to the photostability of the sunscreen agent. It has been demonstrated that the absorption spectra of some UVA and UVB sunscreens decrease as a result of sun exposure when they are conventionally formulated (Gaspar and Maia Campos 2006; Hojerová et al. 2011). The ability of the nanocapsules to enhance the sunscreen photostability has been demonstrated (Weiss-Angeli et al. 2008). The OMC nanoencapsulation improved their photostability when quercetin was co-encapsulated in the formulations. Besides, the chemical stability against the UVA radiation was enhanced when sorbitan monostearate (Span 60®) was used instead of lecithin (Epikuron 170®) as a lipophilic surfactant. The use of sorbitan monostearate leads to a supramolecular architecture named lipid-core nanocapsules, and the use of lecithin (without sorbitan monostearate) is usual to obtain polymeric nanocapsules (Fiel et al. 2011). The antioxidant activity of the quercetin and the capacity of the lipid-core nanocapsules to scatter the light were characteristics that influenced the results. In addition, this kind of polymeric nanocapsule improves transparency of products due to the small size of the particles.

Nanocapsules were also able to protect OMC (Parsol® MCX) and avobenzone (Parsol® 1789) from UV degradation. The increase in the photostability, either when used separately or mixed together, was about 50 % compared to nonencapsulated sunscreens. Nanoemulsions were not capable to protect the sunscreens from UV radiation, showing the advantage of the polymeric wall. Unlike results demonstrated by Weiss-Angeli and coworkers (2008), the addition of an antioxidant, in this case α-tocopherol, does not influence the photoprotection (Hanno et al. 2012).

The better photoprotection was also observed when benzophenone-3 was nanoencapsulated (Paese et al. 2009). An improvement of the effectiveness and of the photoprotection of hydrogels containing benzophenone-3-loaded nanocapsules was observed when benzophenone-3-loaded nanocapsules were compared to gel containing the dispersed sunscreen. In addition, the allergic potential of this formulation was evaluated using the local lymph node and mouse ear swelling tests. After topical application, no allergic reaction was produced in mice, demonstrating the potential use of these nanocapsules for topical administration.

The ability to protect photolabile substances is an important advantage of polymeric nanocapsules. Currently studies are not limited to nanoencapsulated sunscreens but have also considered other substances such as tretinoin, isotretinoin, resveratrol, and clobetasol propionate (Fontana et al. 2009; Ourique et al. 2010; Bettoni et al. 2012; Detoni et al. 2012). The photostability is a consequence of the size, shape, and vesicular structure of nanocapsules. The mean diameter of the nanocapsules, determined by photon correlation spectroscopy, centered between 100 and 500 nm allows the light scattering in the UV region, rendering those nanocarriers satisfactory physical sunscreen with the advantage of transparency after skin application.

Hand hygiene (including handwashing and disinfection) is considered the most important action to control nosocomial infections. Lboutounne and coworkers (2002) have demonstrated the sustained efficacy of chlorhexidine-loaded PCL nanocapsules against *Staphylococcus epidermidis* inoculated onto pig ear skin (Lboutounne et al. 2002). Nhung and coworkers (2007) compared the antibacterial activity of a gel containing chlorhexidine-loaded nanocapsules, named Nanochlorex® (Pirot 2006), to the commercial carbomer gel from Purell®, the Advanced Hand Sanitizer [2-propanol 60 % (v/v) and ethanol 62 % (v/v)]. Nanochlorex® provided

both immediate and sustained antibacterial activity against skin flora. In this work, the sustained efficacy against resident and transient skin flora has been tested either using the in vivo glove juice technique or the ex vivo test which involves repeated contaminations of human skin with *Staphylococcus epidermidis*. The Nanochlorex® did not cause discomfort to the volunteers. In addition, Nanochlorex® had immediate bactericidal efficacy against resident skin flora similar to 2-propanol 60 % (v/v) or to the Advanced Hand Sanitizer (Purell®), while a residual effect has been only observed for Nanochlorex®. The immediate and residual effects were due to the burst release of chlorhexidine (located at the particle–water interface, the polymeric wall) and to the sustained release of the drug from the nanocapsule core with subsequent adhesion to the skin surface, as well as bacteria wall.

Nanocapsules are capable to improve dermatological efficacy and provide a new alternative for the treatment of skin diseases. The potential of a hydrogel containing clobetasol propionate-loaded lipid-core nanocapsules to improve the ecto-nucleoside triphosphate diphosphohydrolase (NTPDase) activity in contact dermatitis model was demonstrated (Fontana et al. 2011). This increase can be related to the better anti-inflammatory activity when clobetasol propionate was nanoencapsulated. Furthermore, similar results observed between untreated and treated groups, using blank nanocapsules (placebo) and clobetasol propionate-loaded lipid-core nanocapsules, indicate that the nanocapsules do not induce inflammatory or immune response, demonstrating the safety of these carriers for cutaneous administration.

In parallel, the nanoencapsulation of natural products has been also studied to improve their biological activities. The hinokitiol, a monoterpenoid (tropolone derivative), obtained from the essential oil of wood of trees in the family Cupressaceae, has an antimicrobial activity also acting as a hair growth promoter (Joo et al. 2008a, b). Hinokitiol-loaded nanocapsules prepared by emulsification–diffusion technique were incorporated into shampoo and hair tonic (Hwang and Kim 2008). The hair growth promotion of the formulations was compared to both a minoxidil solution and saline, as controls. The animals were depilated, treated daily with the different formulations, and evaluated until 21 days. The beginning of hair growth was observed after 12 days, while for the formulations containing the nanocapsules (shampoo and hair tonic), the beginning of hair growth initiated in 7 and 9 days. The digital treatment of the images showed pixel values from 1.5 to 2 times greater for the treated groups (shampoo containing nanocapsules) compared to that of the respective control. Similar increase was also observed for the hair tonic containing nanocapsules. Both cosmetic formulations containing hinokitiol-loaded nanocapsules (shampoo and hair tonic) showed improvement in the hair growth compared to the minoxidil solution.

13.5 Final Remarks

Polymeric nanocapsules represent an interesting carrier system for topical applications. The possibilities of modulating skin penetration/permeation by altering the design of the formulation and the range of applicability are the main subjects under study. Considering the data published up to now, it is possible to affirm that the polymeric wall, surface functionalization, and vehicle have influence on the penetration/permeation of nanoencapsulated active substances.

The polymeric wall influences the penetration/permeation of drugs, as well as its release in the layers of the skin. The polymeric wall may act as a barrier enhancing skin retention, or depending on the material composition of this wall and the active substance, it may increase interaction with the horny layer and facilitate penetration/permeation. Cationic surface functionalization is usually used as a strategy to enhance the interaction of nanocapsules with the skin surface, and as a result the skin retention is improved without enhancing the permeation. Additionally, functionalization with anionic surfactants that act as a permeation enhancer may also facilitate skin penetration. The vehicles used to incorporate nanocapsules are capable of alter-

ing the penetration/permeation of the encapsulated substance. Characteristics such as the presence of absorption promoters and affinity of the substance to the vehicle are very important in determining the influence of the vehicle.

These systems have proved to have various applications for cutaneous treatments by reducing irritant effects and enhancing the drug stability, skin penetration, skin retention, and biological activity of cosmetics and pharmaceuticals.

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Polymeric Micelles in Dermal and Transdermal Delivery

14

Maria Lapteva, Michael Möller, and Yogeshvar N. Kalia

Contents

14.1 Introduction

Topical delivery of drugs directly into the skin offers a targeted approach for the treatment of dermatological conditions and other localized disease states. In addition, transdermal delivery is an option for systemically acting drugs with low oral bioavailability, and it can also offer a more "patient-friendly" alternative to the parenteral route of administration. However, the skin is specifically designed to protect the organism from xenobiotics (Madison 2003). Indeed, only a few drug molecules are able to diffuse passively across the membrane at therapeutic rates, as the skin is naturally refractive to highly hydrophilic or highly lipophilic compounds (Guy 1996). Over the last few decades, various strategies have been investigated in order to overcome the skin permeability barrier which, for most molecules, means its uppermost layer, the *stratum corneum*, can be attributed to the unique structure and composition of this remarkable membrane (Schuetz et al. 2005).

One approach is to encapsulate pharmaceutical active ingredients using small nanometer-sized carriers – in effect, to disguise their unfavorable physicochemical properties – and this is an active area of research in transdermal drug delivery (Schuetz et al. 2005; Cosco et al. 2008; Fireman et al. 2011). The carrier should be able (1) to efficiently encapsulate the desired amount of the drug, (2) to convey the drug to the skin, and (3) to release the drug locally and/or allow its partitioning to deeper tissues and hence the systemic

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circulation. Nanocarriers used to enhance dermal and transdermal delivery are colloids and include liposomes (Pierre and Dos Santos Miranda Costa 2011), vesicles (Sinico and Fadda 2009), transfersomes (Benson 2006), ethosomes (Godin and Touitou 2003), cubosomes (Bender et al. 2005), solid lipid nanoparticles, nanostructured lipid carriers (Muller et al. 2002), polymeric nanoparticles, and finally lipid- and polymer-based micellar solutions. Microemulsions and nanoemulsions can be also cited for their internal nanostructure (Lawrence and Rees 2000; Narang et al. 2007; Azeem et al. 2009).

This chapter deals with the enhancement of dermal and transdermal delivery using polymeric micelles as nanocarriers. The first part discusses some theoretical considerations, and this is followed by an overview of polymers used for micelle preparation with an emphasis on those used in cutaneous drug delivery systems. The next part describes preparation and characterization techniques, and the final section reviews work done to date using polymeric micelles as dermal and transdermal drug carriers and comments on their potential applications.

14.2 Polymeric Micelles: Formation, Structure, and Theoretical Considerations

It is well recognized that surfactants are able to self-assemble into a variety of micro- and nano-sized structures in water (Seddon and Templer 1995; Trickett and Eastoe 2008). At low concentrations, amphiphiles are simply solubilized in water and show no interaction. As their number increases and the concentration exceeds the critical micelle concentration (CMC), then assuming that the critical micelle temperature (CMT) has also been achieved, they spontaneously self-aggregate to form spherical micelles (Fig. 14.1).

Above the CMC, association takes place as the overall free energy of the system decreases since hydrophobic moieties are removed from the aqueous environment and the water hydrogen bond network is reformed (Hiemenz and Rajagopalan 1997; Torchilin 2007; Adams et al. 2003). The micelle is stabilized in aqueous media by the newly formed hydrophobic interactions between the surfactant hydrophobic moieties within its core and interactions with water through its hydrophilic shell (Narang et al. 2007; Torchilin 2007; Jones and Leroux 1999).

Apart from temperature, the CMC can also be influenced by other parameters such as the salt concentration and pH (Hiemenz and Rajagopalan 1997). Amphiphiles of a polymeric nature are of great interest in the drug delivery field because their CMC is usually significantly lower than that of common low molecular weight surfactants, rendering them less sensitive to dilution (Adams et al. 2003). Table 14.1 compares the CMC of some common detergents and block copolymer excipients. Almost all polymeric excipients yield tenfold lower CMC than the low molecular weight detergents.

The size of the micelle is directly dependent on the size and packing of single polymer chains

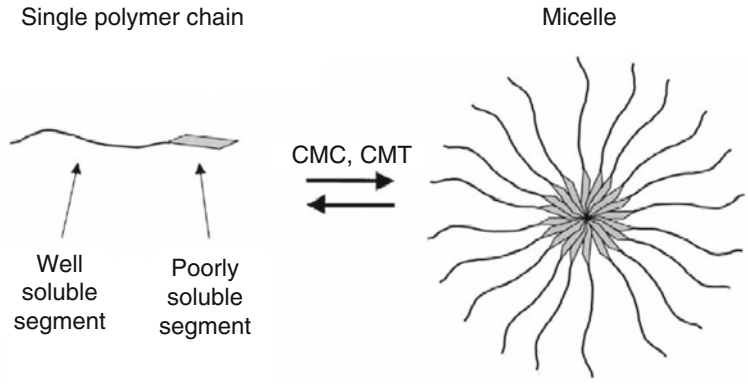


Fig. 14.1 Schematic of a micelle's structure and formation. *CMC* critical micelle concentration, *CMT* critical micelle temperature (Adapted with permission from (Torchilin 2001))

Table 14.1 Examples of experimental CMC for low molecular weight and polymeric excipients

Amphiphile	CMC (M)	Temperature of measurement	Reference
Tween 20	8.04×10^{-5}	RT	Kim and Hsieh (2001)
Tween 80	1.67×10^{-5}	RT	Mandal et al. (1988)
Span 20	1.63×10^{-5}	RT	Kim and Hsieh (2001)
Poly(D,L-lactic acid)/Poly(ethylene glycol)/ Poly(D,L-lactic acid) PLA-PEG-PLA	5.03×10^{-6}	RT	Lee et al. (2006)
Poly(DL-lactic-co-glycolic acid)–poly(ethylene glycol) PLGA-PEG	1.26×10^{-6}	RT	Ashjari et al. (2012)
Pluronic L121 PEO–PPO–PEO (PEO 30 wt. % Mn~4400 Da)	1.00×10^{-6}	37 °C	Lee et al. (2011)
Pluronic P123 PEO–PPO–PEO (PEO 30 wt. % Mn~5800 Da)	4.40×10^{-6}	37 °C	Lee et al. (2011)
Poly(ethylene oxide)-poly(styrene oxide)-poly(ethylene oxide) PEO_{33}–PSO_{14}–PEO_{33}	5.21×10^{-6}	37 °C	Cambon et al. (2012)
Poly(ethylene oxide)-poly(styrene oxide)-poly(ethylene oxide) PEO_{33}–PSO_{10}–PEO_{33}	7.32×10^{-6}	37 °C	Cambon et al. (2012)
Poly(ethylene oxide)-distearoyl-phosphatidylethanolamine PEO $_{(750Da)}$-DSPE	1.0×10^{-5}	n.a.	Torchilin (2007)
Poly(ethylene oxide)-distearoyl-phosphatidylethanolamine PEO $_{(2000Da)}$-DSPE	1.0×10^{-5}	n.a.	Torchilin (2007)
Poly(ethylene oxide)-distearoyl-phosphatidylethanolamine PEO $_{(5000Da)}$-DSPE	6.0×10^{-6}	n.a.	Torchilin (2007)
Poly(ethylene oxide)-dioleoyl-phosphatidylethanolamine PEO $_{(200Da)}$-DOPE	9.0×10^{-6}	n.a.	Torchilin (2007)
Poly(ethylene oxide)-dioleoyl-phosphatidylethanolamine PEO $_{(5000Da)}$-DOPE	7.0×10^{-6}	n.a.	Torchilin (2007)

within the micelle (Hiemenz and Rajagopalan 1997). Generally, micelle sizes can vary from 5 to 100 nm but can be increased by drug incorporation (Jones and Leroux 1999; Torchilin 2007).

14.3 Polymeric Excipients for Micelle Preparation

Polymeric micelles are characterized by the nature of their polymer constituents and composition. Polymers used for micelle formulation are mostly block copolymers that can be subdivided into different classes: (1) diblock copolymers, (2) triblock copolymers, (3) graft copolymers, and (4) star-block copolymers to cite only a few of the most common structures (Torchilin 2001; Kumar et al. 2001). Examples of the different copolymers and the morphology of the resulting micelles can be found in Fig. 14.2.

Diblock A–B copolymers yield micelles when the A and B moieties are, respectively, hydrophilic and lipophilic (Kedar et al. 2010; Gaucher et al. 2005). The hydrophilic part of diblock copolymers is often constituted of poly(ethylene oxide) also called poly(ethylene glycol) (PEO or PEG), a nonbiodegradable yet highly biocompatible polymer (Knop et al. 2010). Other hydrophilic polymers such as poly(N-isopropylacrylamide) (PNIPA) (Xu et al. 2012) or poly(alkylacrylic acid) (PAA) (Chen et al. 1995) can, respectively, provide thermo- or pH-sensitivity to the micelle.

The hydrophobic moieties that can be used are numerous and diverse: they can consist of biodegradable polymers such as poly(lactide) (PLA) (Bachhav et al. 2011), poly(lactic–glycolic acid) (PLGA) (Yoo and Park 2001), and poly (ε-caprolactone) (PCL) (Allen et al. 1998). Polymeric derivatives of amino acids (poly(L-aminoacids)) such as poly(aspartic acid) (Ponta and Bae 2010), poly(b-benzyl L-aspartate) (La et al. 1996), and poly(L-lysine) (Harada et al. 2001) can be cited for their excellent

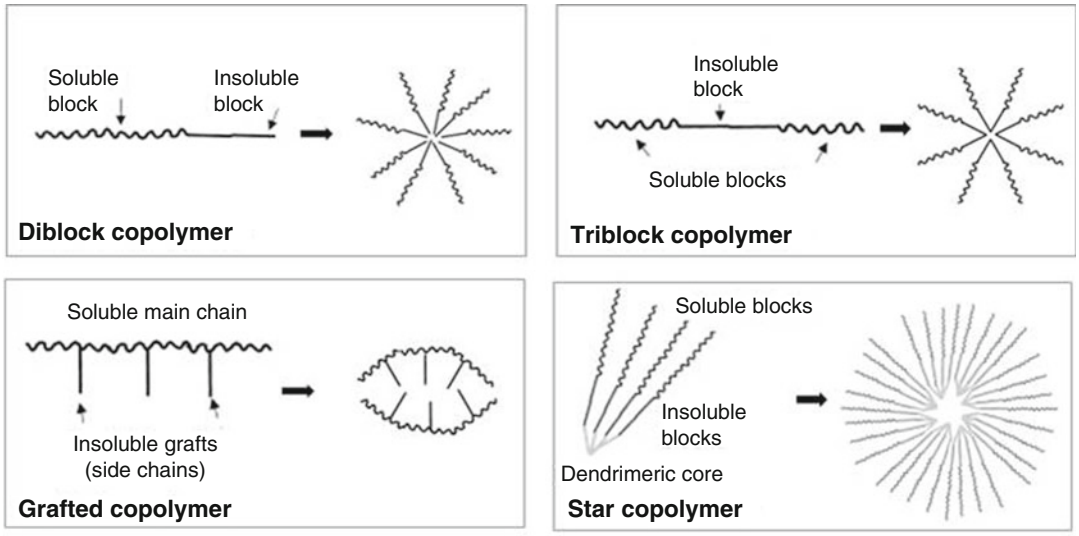


Fig. 14.2 Representation of micelles yielded by diblock, triblock, grafted, and star copolymers (Adapted with permission from (Torchilin 2001))

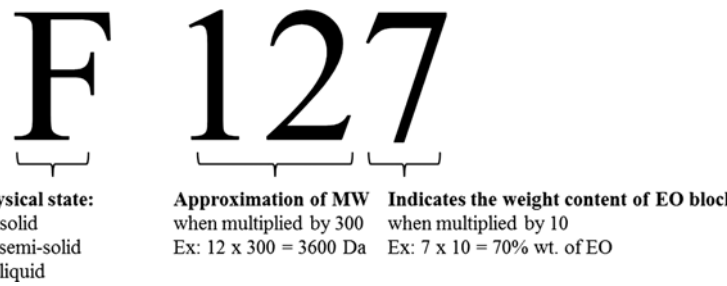


Fig. 14.3 Example of Pluronic® nomenclature interpretation

biocompatibility and degradability; however, the amino acids must present lipophilic properties or rendered hydrophobic by chemical conjugation (Gaucher et al. 2005; Kedar et al. 2010). The lipophilic conjugated moiety can be the drug itself (Li and Kwon 2000). Polystyrene (PS) and poly(methyl methacrylate) (PMMA) are nonbiodegradable hydrophobic polymers that can be used for micelle fabrication (Torchilin 2001; Jones and Leroux 1999). Small alkyl hydrophobic chains (Honeywell-Nguyen et al. 2002a) or diacyl lipids, such as distearoylphosphatidylethanolamine (DSPE) (Sezgin et al. 2006), attached to a water-soluble polymer structure, can also act as the micelle hydrophobic tail.

Triblock A–B–A-type copolymers also exhibit the ability to form micelles (Adams et al. 2003; Attwood et al. 2007; Kabanov et al. 2005) One of the most commonly used copolymers is poly(ethylene oxide)–poly(propylene oxide)–poly(ethylene oxide) (PEO–PPO–PEO; also referred to as poloxamer (Pluronic®, BASF, Germany)). The Pluronic® nomenclature itself provides a precise description of the copolymer characteristics (Kabanov and Alakhov 2002); Fig. 14.3 presents a guide to interpreting the nomenclature taking Pluronic® F 127 as an example.

The hydrophobic PPO moiety constitutes the core of the micelle, whereas the PEO parts form its hydrophilic shell. In addition to forming micelles above the CMC, poloxamers also demonstrate a remarkable temperature-dependent phase behavior (Fig. 14.4). Thus, when a micelle solution is heated up to 25–37 ° C, a phase transition towards a gel occurs (Attwood et al. 2007).

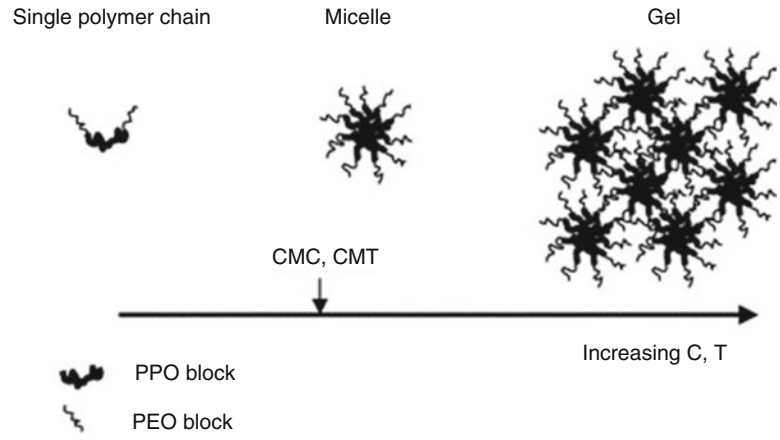


Fig. 14.4 Micellization and gelation of Pluronic® PEO–PPO–PEO copolymer. *CMC* critical micelle concentration, *CMT* critical micelle temperature (With permission from (Kabanov et al. 2005))

This excipient is of considerable interest for pharmaceutical applications because the fluid micelle formulation can be injected, and then as it is warmed to body temperature, this triggers *in situ* gelation thus providing controlled release of the loaded drug (Attwood et al. 2007).

Other triblock copolymers can be formed using the various hydrophobic and hydrophilic chains mentioned above.

In the case of graft copolymers, the hydrophobic chains are randomly distributed along a hydrophilic chain (Fig. 14.2). As the hydrophobic fragments of graft copolymers have decreased mobility, this facilitates the aggregation of graft-copolymer micelles, leading to lower CMC and aggregation numbers. For this reason, these micelles can also have smaller sizes (Torchilin 2001).

Star-block copolymers have also attracted attention in the field of drug delivery. Their more complex architecture consists of several linear diblock "arms" radially arranged around a central core (Fig. 14.2). Compared to conventional linear block copolymers, star-block copolymers can also exhibit better aggregation properties due to the packing of the polymer chains and small hydrodynamic volume (Petrova et al. 2012; Poree et al. 2011).

It should be mentioned that the micelle-based formulations prepared with the above copolymers were mainly developed for parenteral and oral administration. Despite the success of nano-sized delivery systems for dermal and transdermal delivery, the potential of polymeric micelles in this field has not been extensively studied. Table 14.2 presents some copolymers that have been used to date for the formulation of micelles in skin penetration studies.

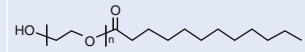
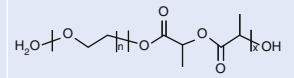
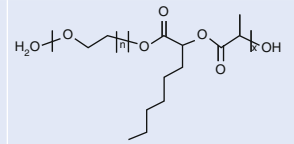
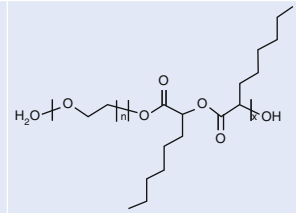
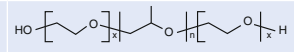
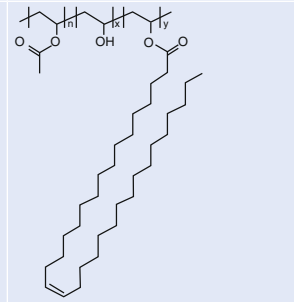
14.4 Drug Incorporation and Micelle Characterization

14.4.1 Drug Solubilization

One of the essential properties of polymeric micelles for pharmaceutical applications is their ability to solubilize sparingly water-soluble drugs within their core (Torchilin 2001; Narang et al. 2007; Mondon et al. 2008). Lipophilic drugs partition into the central core of the micelle, whereas less lipophilic compounds can be located partly in the core and the shell of the micelle or entirely in the shell for hydrophilic compounds (Fig. 14.5).

The ability of the micelle to solubilize lipophilic compounds directly depends on the nature and the size of the hydrophobic moiety of the copolymer. The nature can influence the intermolecular interactions between the drug and the polymer, and the size will affect the amount of drug that can be incorporated. Conversely, the drug itself can impact the characteristics of the micelle, for example, leading to a swelling of its core (Torchilin 2007). Regarding skin delivery, the enhancement of drug solubility in the

Table 14.2 Examples of polymers used for deriving micelle-based formulations for skin drug delivery

Polymer type	Denomination of the copolymer	Structure of the copolymer	Reference
PEO derivative lauric acid	PEG-8-L (octaoxyethylene laurate ester)		Honeywell-Nguyen et al. (2002a, b, c, 2003)
Diblock	MPEG-PLA methoxy-poly(ethylene glycol)-polylactide[a]		Bachhav et al. (2011)
Diblock	MPEG-hex PLA methoxy-poly(ethylene glycol)-hexyl substituted polylactide[a]		Bachhav et al. (2011)
Diblock	MPEG-dihex PLA methoxy-poly(ethylene glycol)-dihexyl substituted polylactide[c]		Bachhav et al. (2011)
Triblock	PEO–PPO–PEO poly(ethylene oxide)–poly(propylene oxide)–poly(ethylene oxide)		Liaw and Lin (2000), Zheng et al. (2010)
Triblock	Poly(vinylalcohol-co-vinyloleate)		Luppi et al. (2002)
Star	Amphiphilic star block copolymers	(See Fig. 14.12 for structure)	Poree et al. (2011)

[a]Methoxy-poly(ethylene glycol)-poly(lactic acid)
[b]Methoxy-poly(ethylene glycol)-poly(lactic acid co-hydroxyoctanoic acid)
[c]Methoxy-poly(ethylene glycol)-poly(lactic acid co-2-hydroxyoctanoic acid)

micelle-based vehicle enables higher drug loading and the potential for increased cutaneous bioavailability.

14.4.2 Methods of Preparation

Depending on the physicochemical properties of the drug/copolymer, several preparation methods can be used. The most common methods are direct dissolution, dialysis, solvent evaporation, oil-in-water emulsion, solid dispersion, and freeze-drying (Kedar et al. 2010; Gaucher et al. 2005). In addition to controlling the micelle size, the choice of the preparation method can have a significant impact on the drug loading in the micelle.

14.4.2.1 Direct Dissolution

This method consists of dissolving both the block copolymer and drug in the aqueous media. It is suitable when both the copolymer and the drug are relatively hydrophilic but results in low drug loadings (Jones and Leroux 1999). Moreover, the micellization may require the temperature to be increased in order to dehydrate the lipophilic parts of the polymer (Gaucher et al. 2005).

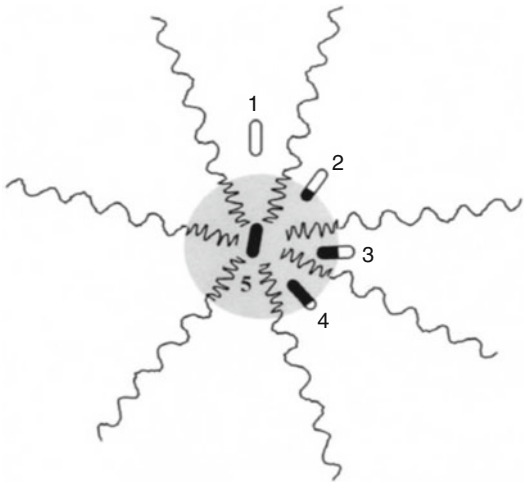


Fig. 14.5 Theoretical location of the drug molecules within the micelle structure according to their lipophilicity. *Grey-black*, lipophilic part; *white*, hydrophilic part. Example: case 3 is an amphiphilic drug molecule and can interact with both core and shell of the micelle. *1*, *2*, *3*, *4*, *5* represent theoretical drug location inside the micelle depending on theit properties: *1* for the most hydrophilic compoum and *5*, formost lipophilic (Adapted with permission from (Torchilin 2001))

14.4.2.2 Dialysis

The dialysis method uses water miscible organic solvents such as dimethyl sulfoxide, N,N-dimethylformamide, acetonitrile, tetrahydrofuran, or acetone in order to co-solubilize the polymer and the drug. The organic solvent is finally dialyzed against water through a semipermeable membrane. The slow removal of organic solvent decreases the solubility of the copolymer and the drug resulting in triggering of self-assembly to form micelles (Gaucher et al. 2005; Kedar et al. 2010).

14.4.2.3 Solvent Evaporation

This method utilizes water miscible solvents with low boiling points. The copolymer and the drug are both present in the organic solution which is introduced under sonication or agitation into the aqueous phase. The organic solvent is then removed by evaporation under reduced pressure leaving loaded micelles in the aqueous solution (Bachhav et al. 2011; Gaucher et al. 2005; Kedar et al. 2010).

14.4.2.4 Oil-in-Water Emulsion

In the case where neither the drug nor copolymer is soluble in water miscible organic solvents, the oil-in-water emulsion method can be used. The components are dissolved in water-immiscible solvents, for example, chloroform, and added under stirring in the aqueous phase in order to form an oil-in-water emulsion. The copolymer, due to its amphiphilic properties, is located at the interface. The organic solvent is finally evaporated allowing the "oil" globules to shrink until the formation of solvent-free solution containing micelles (Gaucher et al. 2005; Kedar et al. 2010).

14.4.2.5 Solid Dispersion (Solution Casting)

In this method, the copolymer and the drug are dissolved in an appropriate volatile solvent. After evaporation of the solvent to dryness, a polymeric drug-containing matrix is obtained. Micelles are formed after addition of hot water to this preheated matrix (Gaucher et al. 2005; Kedar et al. 2010; Zhang et al. 1996).

14.4.2.6 Freeze-Drying

Here, both the copolymer (PVP-*b*-PDLLA) and the drug are dissolved in a water/tert-butanol mixture and undergo lyophilization. Micelles formed spontaneously when the freeze-dried polymer–drug cake was reconstituted with water (Fournier et al. 2004; Gaucher et al. 2005).

It is important to emphasize that the method of preparation should ensure the nontoxicity of the formulation, that is, if the use of an organic solvent cannot be avoided, it has to be selected from class 2 or 3 US Pharmacopeia categories and the residual amount in the formulation must be assessed and be acceptable for the specific applications.

14.4.3 Typical Characteristics of Micelle Formulations

14.4.3.1 Critical Micelle Concentration

The CMC is a critical parameter for the stability of micelle formulations since they will disassemble below this concentration – this can occur by

simple dilution in which case poorly water-soluble drugs can precipitate out of solution. The transition from a surfactant solution to surfactant micellar aggregates is accompanied by a sudden change in the physical characteristics of the media such as the osmotic pressure or conductivity (Hiemenz and Rajagopalan 1997). However, the most commonly used method to determine the CMC of polymeric micelles is the fluorescent dye solubilization method. This technique utilizes lipophilic dyes such as pyrene (Cambon et al. 2012), 1-pyrenehexanoic acid (Lee et al. 2006), and Nile red (Trimaille et al. 2006) and relies on the fact that the fluorescent dye is water insoluble and shows low fluorescence below the CMC, whereas above the CMC, it is solubilized inside the micelle leading to an increase in fluorescence intensity and/or modified fluorescence spectrum. The fluorescence intensity is reported as a function of copolymer concentration, and the inflection point of the sigmoid is considered to denote the CMC (Torchilin 2001; Kedar et al. 2010) (Fig. 14.6).

14.4.3.2 Drug Content

As mentioned above, micelles can facilitate the dissolution of poorly water-soluble drugs in aqueous formulations with high efficiencies. Much higher drug concentrations can be achieved in the formulation with less excipient, and this can help to deliver greater amounts of drug (greater delivery efficiency can help to decrease the dose that needs to be administered).

The ability of micelles to incorporate a drug can be assessed by different analytical methods after its release from the micelles by dilution in an appropriate solvent. Drug loading can be calculated according to Eq. 14.1, and incorporation efficiency can be calculated according to Eq. 14.2.

$$\text{Drug loading (\%)} = \frac{\text{mass of the drug incorporated (g)}}{\text{mass of the copolymer used} + \text{mass of the drug incorporated (g)}} \times 100 \quad (14.1)$$

$$\text{Incorporation efficiency (\%)} = \frac{\text{mass of the drug incorporated (g)}}{\text{mass of the drug introduced (g)}} \times 100 \quad (14.2)$$

It should be noted that depending on the preparation method, the micellar formulation may be at saturation thus leading to drug precipitation after equilibration thus impacting on the drug loading and incorporation efficiency.

14.4.3.3 Micelles as Nano-sized Drug Carriers

It has been suggested that the size of a carrier is of major importance, for example, in the case of follicular targeting, it can influence the depth of penetration into the follicular duct (Neubert 2011). Micelles typically have sizes below 100 nm, but a more accurate estimation of the exact diameter can be of interest.

The most commonly used analytical method for micelle size characterization is dynamic light scattering (DLS), also known as correlation spectroscopy. The particles in solution scatter the incident laser beam. The Brownian motion of the particles means that the intensity of the scattered light is constantly fluctuating. These fluctuations are converted into electrical pulses, which are fed into a digital correlator. An autocorrelation function allows the particle size to be deduced as it is inversely related to its velocity in solution (Kedar et al. 2010).

14.4.3.4 Micelle Morphology

Depending on the block sizes of the block copolymers used, different micelle morphologies can occur ranging from homogenous round/spherical micelles to slightly elongated wormlike micelles. It has been shown that the shape of silica nanoparticles can influence the cellular uptake mechanisms (Herd et al. 2013). By analogy, micelle

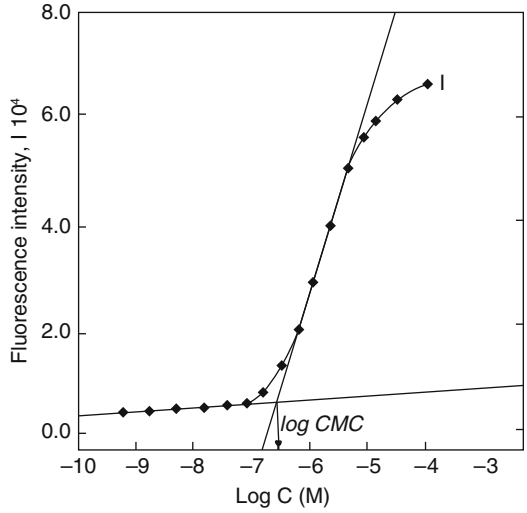


Fig. 14.6 Typical curve showing the evolution of the fluorescent signal as a function of copolymer content (logarithmic scale). The increase in dye fluorescence (inflection point indicated by the *arrow* on the graph) denotes its solubilization inside the micelles and thus the CMC (Adapted with permission from (Torchilin 2001))

morphology could be of importance in the skin penetration.

Micelle morphology can be evaluated using microscopic techniques. While conventional optical microscopy lacks resolution, micelles can be observed by transmission electron microscopy (TEM), scanning electron microscopy (SEM), or atomic force microscopy (Kedar et al. 2010). However, the micelles can be damaged or undergo aggregation during the sample preparation procedure and thus bias the microscopic observation (Krauel et al. 2007).

14.5 Polymeric Micelles in Skin Drug Delivery

Studies have shown that polymeric micelle-based delivery systems are efficient for parenteral use. Moreover, the potential of colloidal nanocarriers for dermal and transdermal has also been demonstrated. Despite this, investigations describing the application of polymeric micelles for delivery into and across the skin are rare.

One of the first studies to test the potential of polymeric micelles for skin application investigated the transdermal administration of fentanyl, an analgesic and anesthetic agent, incorporated in PEO–PPO–PEO-based formulations (20 % PPO; 80 % PEO; MW = 8400 Da), and was tested across the nude mouse skin *in vitro* and *in vivo* in New Zealand rabbits (Liaw and Lin 2000). The copolymer was found to form micelles above a concentration of 0.1 % (CMC) yielding small 50 nm micelles. *In vitro*, increase of the copolymer content to 46 % resulted in a pseudo-zero-order sustained release of fentanyl compared to the drug solution (apparent permeability coefficients of $0.13 \pm 0.02 \times 10^{-6}$ and 2.24 ± 0.47 cm/s, respectively). This was explained by the progressive gelation of the vehicle. The 46 % PEO–PPO–PEO formulation was then selected to develop a patch for a preliminary kinetics study *in vivo*. Steady-state plasma levels were reached within 24 h after patch application and were maintained for 72 h. The copolymer was detected in the receiver compartment at concentrations above the CMC, demonstrating that it was able to cross the nude mice skin. However, it was not possible to determine whether single copolymer chains passed through the skin and reformed micelles in the receiver compartment or the intact micelle transited the skin. Inspired by the works of Cevc et al. on ultra-flexible vesicles (Cevc et al. 1998), the authors hypothesized that the micelle could penetrate through the skin because of its small size and by means of micelle deformation. This hypothesis was supported by the fact that the fentanyl half-life *in vivo* using PEO–PPO–PEO copolymer gel was longer than the one after I.V. injection, suggesting that *in vivo* fentanyl was still protected by being covered in the micelle core.

In our view, the experimental method to detect the presence PEO–PPO–PEO copolymer in the receiver compartment does not seem to be reliable. It involved the addition of a known amount of pyrene (6×10^{-7} M) to a sample of receiver media and followed by analysis by fluorescence spectroscopy. The ratio between the I_1 and I_3 peaks in the pyrene fluorescence spectrum was calculated and was found to decrease with per-

meation time. The authors concluded that this indicated the transfer of the dye into a hydrophobic environment, that is, the micelle. However, it has to be pointed out that a hydrophobic environment in the receiver compartment can also be due to the release of some amphiphilic endogenous compounds in the skin, for example, aggregated proteins (Hawe et al. 2008); these could modify the pyrene emission spectrum, and this could be incorrectly interpreted as indicating the presence of polymeric micelles. Moreover, the increased half-life of fentanyl observed in rabbits could be explained by a reservoir function of the *stratum corneum*, prolonging the drug elimination phase. In conclusion, the PEO–PPO–PEO copolymer gel seems to be a promising vehicle for controlled delivery of drugs through the skin, but the delivery mechanism needs to be further investigated.

Interesting studies were performed by Bouwstra et al. (Honeywell-Nguyen et al. 2002a, b, c, 2003). Although their investigations were mainly focused on the development of more or less flexible vesicles for cutaneous drug delivery, some very useful findings were made about micelles as well.

In these studies, micelles constituted of a PEO derivative of lauric acid: PEG-8-L (octaoxyethylene laurate ester), served as control formulations, while the vesicles contained PEG-8-L, sucrose laurate ester (L-595) and sulfosuccinate in various proportions. In 2002, Honeywell-Nguyen et al. investigated the delivery of lidocaine, pergolide, and rotigotine from vesicles and micelles (Honeywell-Nguyen et al. 2002b, c, 2003). For all tested drugs, delivery across the human skin from elastic vesicles was significantly higher than from rigid vesicles. Micelles yielded an intermediate delivery. The penetration mechanisms have been investigated by van den Bergh et al. using electron microscopy and two-photon confocal microscopy (van den Bergh et al. 1999) and by Honeywell–Nguyen (Honeywell-Nguyen et al. 2002a) using tape-stripping and freeze-fracture electron microscopy (FFEM).

In the first study (van den Bergh et al. 1999), fluorescein-DHPE, used as a model dye, showed a homogeneous intercellular penetration with PEG-8-L micelles, whereas elastic vesicles were retrieved in channel-like structures. In the second study (Honeywell-Nguyen et al. 2002a), again, vesicular structures were found to accumulate in the channel-like regions up to the ninth tape-strip (Fig. 14.7), while micelles were present as "rough, irregular structures" covering large areas of the surface of SC but less seen in deeper layers (Fig. 14.8).

From Fig. 14.8, it can be observed that the 15th strip of the skin treated with micelles has a "rough and irregular appearance" compared to 15th strip of the skin treated with control buffer. The authors suggested that micelles induce obvious changes in the *stratum corneum* lipids, thus acting as penetration enhancers.

From these findings, one may conclude that just like vesicles, micelles have the potential to enhance skin delivery of drugs; however, in contrast to elastic vesicles, the PEG-8-L micelle does not seem to penetrate the skin in its intact form.

In 2002, Luppi et al. (2002) published a study on poly(vinylalcohol-co-vinyloleate) micelles for the transcutaneous delivery of retinyl palmitate. Micelles with different drug loadings ranging from 200 to 1000 mg of retinyl palmitate per gram of copolymer were studied. The micelles

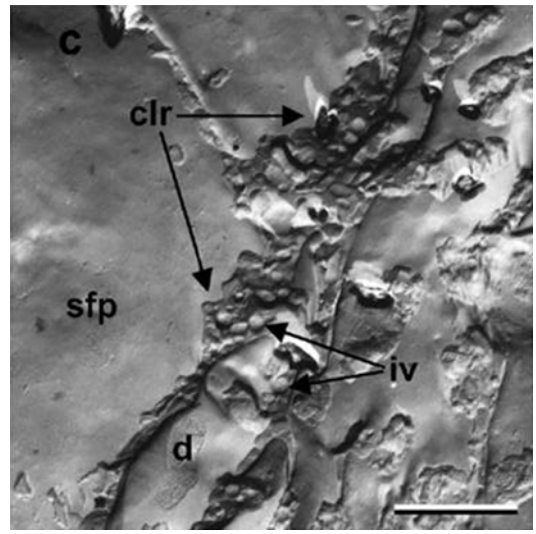


Fig. 14.7 FFEM micrograph of the 9th tape strip of the skin treated with elastic vesicles. *Abbreviations*: *sfp* smooth fracture planes, *d* desmosome, *clr* channel-like regions, *iv* intact vesicles. Scale bar = 1 μm (Adapted with permission from (Honeywell-Nguyen et al. 2002a))

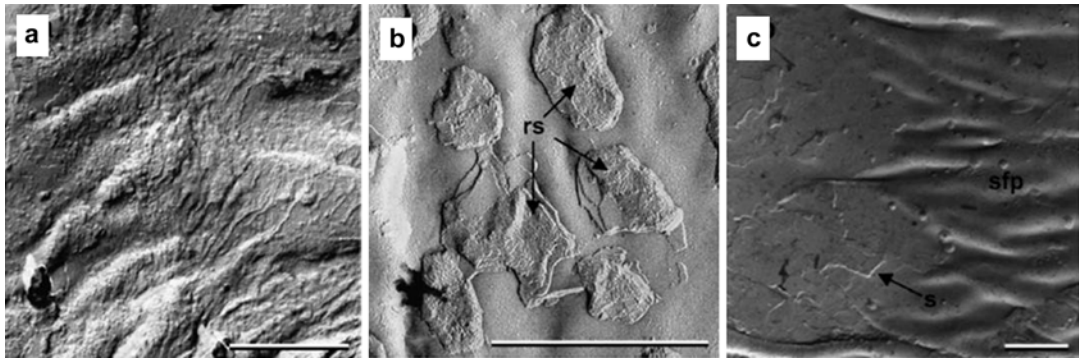


Fig. 14.8 FFEM micrograph of (**a**) 1st tape strip of the skin treated with PEG-8-L micelles, (**b**) 15st tape strip of the skin treated with PEG-8-L micelles, and (**c**) 15st tape strip of the skin treated with control buffer. *Abbreviations*: *rs* rough surfaces, *sfp* smooth fracture planes. Scale bar = 1 μm, *s* steps due to fracturing across an intercellular lipid-layer (Adapted with permission from (Honeywell-Nguyen et al. 2002a))

had sizes of more than 200 nm, and this might have been due to the length of the hydrophobic moiety of the copolymer or to the use of ethylene glycol dimethyl ether as a solubilizer for drug and copolymer. However, the authors were able to correlate the enhanced skin delivery from micelles with their size, suggesting that the smaller the micelle, the greater the delivery. Although this could have been a very interesting conclusion, this study does have some flaws. First of all, the porcine ear skin used in the study was left to hydrate for 24 h before the experiments, which could have compromised the skin barrier. Second, ethylene glycol dimethyl ether (class 2 in USP residual solvents classification) was used as the solubilizer for the drug and the copolymer during micelle preparation but was not subsequently eliminated from the formulation. This is undesirable for two reasons: (1) it is not tolerated by the skin, and (2) it can affect the results since it can also serve as a penetration enhancer. Finally, the authors claim to have measured the transcutaneous permeation of the drugs, but only drug deposited in the skin was quantified. Consequently, few conclusions can be drawn from the study.

More recently, Zheng et al. (2010) tried to encapsulate honokiol, a poorly water-soluble lignan extracted from *Magnolia* species and presenting various interesting pharmacological properties. The PEO–PPO–PEO Pluronic® F127 (Sigma-Aldrich, China) copolymer was used to formulate micelles using the direct dissolution method assisted by ultrasound, thus avoiding the use of toxic organic solvents. Micelles presented particle sizes of ~30 nm and had high entrapment efficiency. When the formulation (20 % of F127) was placed at 37 °C, a transition to the gel phase occurred. The sol–gel transition temperature was found to be linearly dependent on the drug content; thus, as drug was released from the formulation, the gel was thinning. The release was found to follow zero-order kinetics. According to the permeation profile, up to 80 μg/cm^2 was delivered in 24 h through the abdominal skin of Sprague–Dawley rats. Yet, because of the absence of a control formulation, the authors could not comment on the eventual delivery enhancement or mechanism of penetration.

In an interesting study by Bachhav et al. (2011), three topically administered antifungal drugs (clotrimazole, fluconazole, and econazole nitrate) were successfully formulated with three different copolymers of the same molecular weights (approximately 5000 Da): methoxy-poly(ethylene glycol)-polylactide (MPEG-PLA); methoxy-poly(ethylene glycol)-hexyl substituted polylactide (MPEG-hexPLA), and methoxy-poly(ethylene glycol)-dihexyl substituted polylactide (MPEG-dihexPLA) (see Table 14.2). Micelles were prepared using the solvent evaporation method and demonstrated spherical morphology and small particle size (Fig. 14.9).

For these different polymers, the increasing degree of substitution of the methyl groups of the standard poly (lactide) by hexyl groups conferred an increasing lipophilicity to the core of the micelle. The best incorporation efficiency reached 98.3 % and was provided by the MPEG-dihexPLA micelles loaded with econazole. Therefore, the *in vitro* delivery experiments were performed with this formulation.

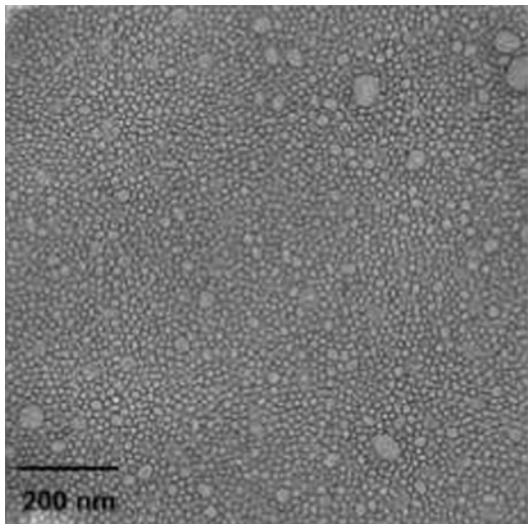


Fig. 14.9 TEM picture of MPEG-dihexPLA micelles loaded with econazole (Adapted with permission from (Bachhav et al. 2011))

The 1 % econazole nitrate micelle formulation was evaluated against the commercially available liposomal formulation: Pevaryl® 1 % econazole nitrate liposomal gel (Janssen-Cilag AG, Baar, Switzerland). The *in vitro* skin deposition from the polymeric micelles was significantly higher than that from Pevaryl® liposomal gel in both the porcine and human skin (Fig. 14.10), while no permeation of the drug was observed.

The authors concluded that despite the commercial liposomal formulation containing numerous penetration enhancers such as linalol, cinnamic alcohol, cinnamic aldehyde, and Oleoyl macrogol-6-glycerides (Labrafil M 1944 CS, Gattefossé), the micelle formulation yielded better skin delivery due to the smaller size of the nanocarriers. Also, this performance was proposed to be influenced by the low glass transition temperature of the copolymer (T_g), suggesting that the micelle core remained in its viscous state and yielded flexible micelle structures, thus producing a better interaction of the micelle constituents with *stratum corneum*.

Finally, the authors investigated micelle penetration pathways with confocal laser scanning microscopy (CLSM) using fluorescein. CLSM study confirmed that the micelles are able to deliver high amounts of the dye to the skin compared to the control aqueous fluorescein solution (Fig. 14.11).

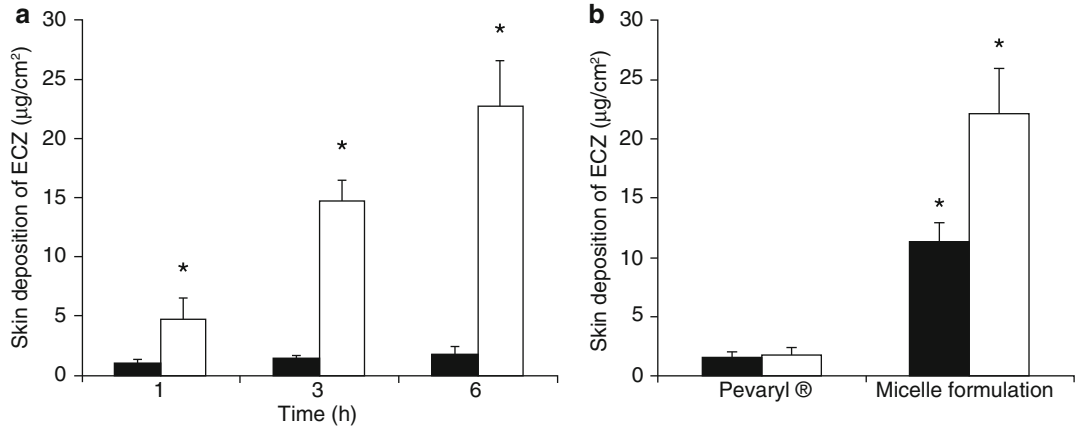


Fig. 14.10 (**a**) Skin deposition of econazole nitrate from the MPEG-dihexPLA micelles (□) and Pevaryl® liposomal gel (■) in function of time. (**b**) Comparison of econazole nitrate delivery into the porcine skin (□) and human skin (■) from Pevaryl® liposomal gel and micelle formulation (Adapted with permission from (Bachhav et al. 2011))

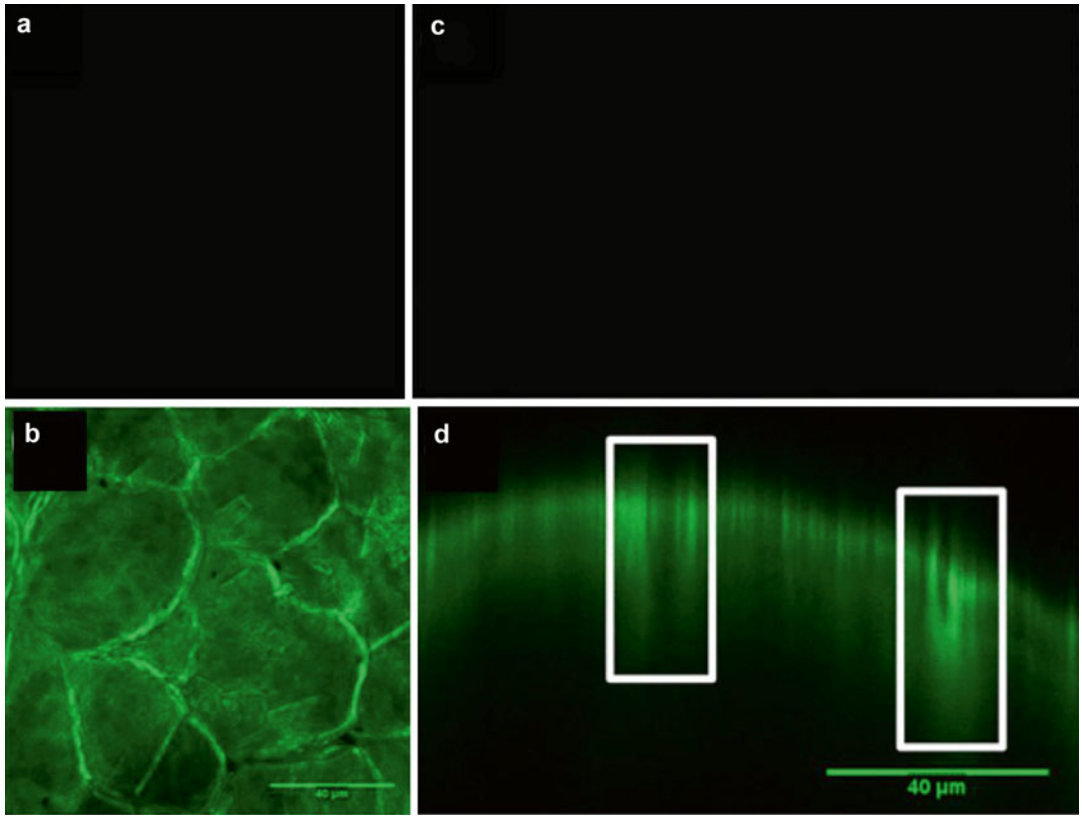


Fig. 14.11 CLSM images of the porcine skin after 24 h micelle or control formulation application. (**a**) XY-plane of the skin treated with fluorescein control solution, (**b**) XY-plane of the skin treated with micelle fluorescein formulation, (**c**) XZ-section of the skin treated with fluorescein control solution, (**d**) XZ-section of the skin treated with micelle fluorescein formulation (Adapted with permission from (Bachhav et al. 2011))

Moreover, it was observed that appendageal skin structures (white squares in Fig. 14.11) exhibited stronger fluorescence suggesting that micelles could penetrate preferentially via these penetration pathways.

Poree et al. (2011) developed amphiphilic star-block copolymer to deliver two fluorescent dyes into the porcine skin: proflavin or rhodamine B. The amphiphilic star-block copolymers were constructed so as to form reverse micelles (Fig. 14.12). The hydrophilic core was constituted by an oligo (ethylene glycol) methacrylate polymer (OEGMA block), and the hydrophobic shell consisted of the lauryl methacrylate polymer (LMA block). This diblock copolymer was grafted either on 1, 6, or 12 arms of the 2, 2-bis(hydroxymethyl) propionic acid-based brominated dendritic core. The resulting amphiphilic star copolymers could aggregate to form reverse micelles (Fig. 14.12).

TEM and DLS were used to characterize the reverse micelles. The linear 1-arm amphiphilic polymer exhibited large spherical aggregates between 175 and 250 nm, whereas the 6- and 12-arm micelles exhibited smaller particle sizes (44–53 and 42–55 nm, respectively). To explain the difference in size, the extended chain lengths of the copolymers were approximated using molecular modeling. According to the modeling data, the chain of the 1-arm copolymer was more extended than that of the 12-arm copolymer (8–10 and 5–7 nm, respectively). The extended chain length of the micelle constituent directly influences its size. Finally, the dye solubilization experiment showed that 6- and 12-arm star copolymers exhibited a lower CMC than the 1-arm

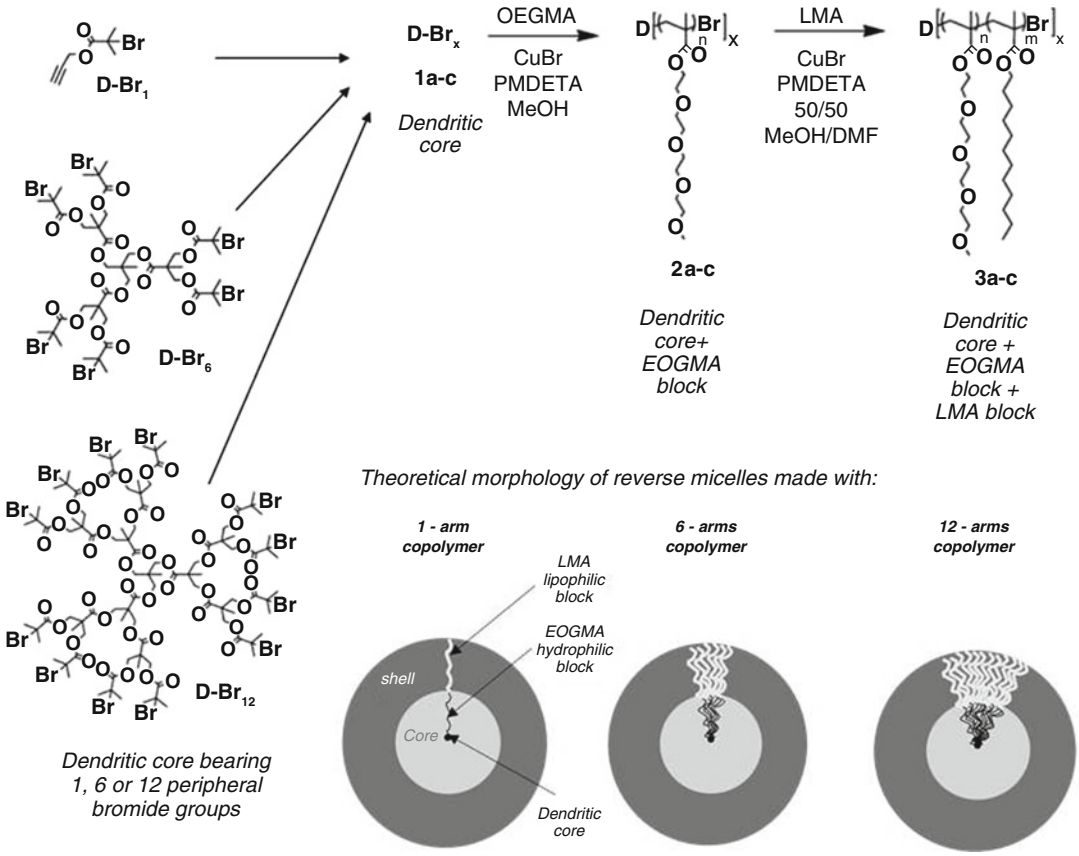


Fig. 14.12 Synthesis of amphiphilic star-block copolymers and their aggregation into reverse micelles (Adapted with permission from (Poree et al. 2011))

star copolymer and a better ability to encapsulate polar dyes.

The skin permeation experiments and visualization of the skin by microscopy demonstrated that the micelles formed by the 12-arm star copolymers induced a better penetration of both rhodamine and proflavin, allowing them to reach the epidermis (Fig. 14.13).

The authors suggested that the skin penetration enhancement yielded by 12-arm star copolymers was due to their smaller size, lower aggregation numbers, and increased loading. Despite the fact there was no significant difference in micelle size between the 6- and 12-arm star copolymers, the two formulations demonstrated very different penetration profiles. Therefore, the ability of the 12-arm star copolymer to encapsulate a greater amount of dye was probably the main explanation for the results.

It has to be mentioned that fluorescence microscopy techniques are very informative about the localization of the encapsulated dye in the skin; however, the question of the localization of the polymer remains unanswered. Fluorescence microscopy might also help to determine whether the polymer remains on the skin or surface or if it enters the membrane.

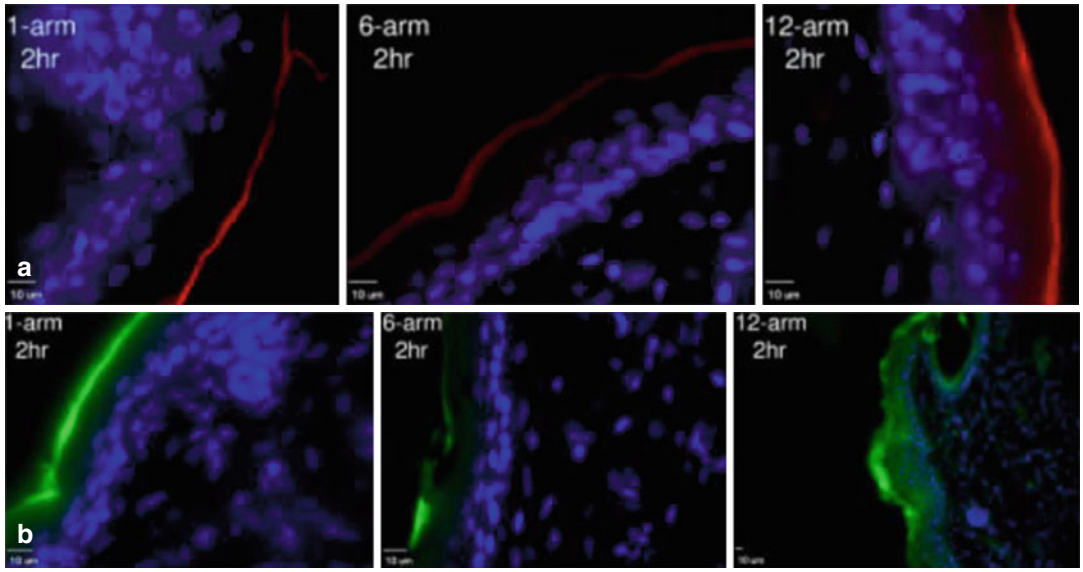


Fig. 14.13 Microscopy images of the skin cross-section. (**a**) Micelles encapsulating rhodamine, (**b**) micelles encapsulating proflavin. Nuclei have been stained with DAPI (Poree et al. 2011))

Conclusion

Many drugs intended for topical and transdermal application suffer from poor bioavailability when administered using conventional vehicles. Although studies into the ability of polymeric micelles to enhance dermal and transdermal drug delivery are not numerous, the work to date clearly demonstrates that micelles are efficient drug delivery systems for cutaneous application. Thus, these carriers may enable targeted drug delivery and lead to optimized disease management.

Since nanoparticles were first applied on the skin as drug carriers, the question of whether or not these nano-sized drug carriers can cross the *stratum corneum* has been and is still a matter for debate. In the case of micelles, authors of early studies indeed reported finding "intact micelles" in receptor media after the permeation experiment (Liaw and Lin 2000). However, subsequent investigations did not evidence any micelle penetration to deep layers or across the skin. On the contrary, it is supposed that the micelle can disaggregate upon contact with the skin because of polarity changes (Poree et al. 2011), and its constituents can then act as penetration enhancers and disturb the packing of intercellular lipids (Honeywell-Nguyen et al. 2002a). Finally, recent studies indicate that micelles due to their small size might enhance drug delivery by having a larger contact surface with the skin or by the formation of a depot film on the skin surface and in its appendages (Bachhav et al. 2011). Despite the numerous hypotheses on the possible interactions between micelles and the skin, in general most authors agree that the potential of polymeric micelles to enhance cutaneous delivery is also due to their ability to incorporate high amounts of drug and thus to increase thermodynamic activity.

The findings about the role of polymeric micelles in drug delivery to the skin to date

seem to be coherent and promising. More investigations on the potential of these remarkable carriers should be conducted especially as knowledge in polymer science is constantly growing and the use of many existing biocompatible copolymers has not yet been tested on the skin.

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Core-Shell Nanotransporters for the Skin

15

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15.1 Introduction

Since drug penetration into human skin amounts only to a few percent of the applied dose, various approaches are investigated for penetration enhancement. Nanoparticles of various types can enhance the notoriously poor skin penetration of drugs, yet different carriers have been rarely compared and differences in efficiency are essentially unknown. Moreover, relevant properties of the carrier (e.g., size, rigidity, aggregation, overall/surface charge and hydrophobic-hydrophilic characteristics), the vehicle and the loaded drugs are taken into account only recently. In accordance with the intensely studied targeted delivery of antitumor drugs applied by the i.v. route, these characteristics of a nanoparticle system should be of relevance, too, for drug delivery to the skin following topical application.

In the 1980s lipid carriers have been a major focus of research in drug delivery to the skin. Pharmaceutical use of liposomes and other lipid nanoparticles, however, is still limited due to an often insufficient stability which holds true especially for liposomes. An alternative for those nanoparticles being aggregates of individual lipid molecules are proteins or – more likely – other large synthetic molecules, the single molecule being nanosized. Single-molecule nanoparticles can overcome the limited stability of lipid aggregates. Drugs or – for experimental purposes – probes (dyes, spin probes) can be supramolecularly

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complexed or covalently bound to the nanoparticles being these aggregates or single molecules.

In skin diseases the impaired stratum corneum barrier may allow the penetration of nanoparticles which do not surmount the intact barrier. Thus with large synthetic single-molecule drug carriers adverse effects need to be addressed most carefully. Lipid particles are less problematic than the polymer particles as the aggregates disintegrate at the skin surface and intact particles do not penetrate the skin. For instance, solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC) can gelify on the skin surface or interact with skin surface lipids and form an occlusive film, whereas conventional liposomes dehydrate, coalesce, and fuse on the outer first cell layer of the skin. Specially engineered liposomes, however, can even reach the normal viable epidermis. Ethosomes and invasomes can surmount the intact stratum corneum by virtue of their highly fluid bilayers that contain a permeation enhancer, ethanol, and terpenes, respectively. Similarly, the highly deformable Transfersomes™ (Idea AG, Germany) are reported to permeate the intact stratum corneum across hydrophilic nanochannels. All these lipid aggregates are devoid of adverse effects after repeated application (Romero and Morilla 2013). The lipids often are physiological compounds or closely related structures which are degradated by physiological pathways of lipid metabolism.

Besides the safety of the intact single-molecule carriers, risks induced by the degradation products have to be considered. For example, polylactic acid (PLA) and poly(glycolic-co-lactic) acid (PGLA) polymer-based carriers may be subject to local degradation resulting in the release of acids. Esterases are abundant in human skin and efficiently cleave ester drugs (Bätz et al. 2013). In healthy skin, pH is highly regulated to around pH 5.5 (Schmid-Wendtner and Korting 2006) and the influence of released acid is unknown. Well-tolerated alternatives to PLA/PLGA nanoparticles have been looked for. As known from i.v. studies, functionalization of the particle surface in particular by polyethylene glycol (PEG) can improve both stability and tolerability of various nanoparticles, e.g., liposomes and dendrimers. PEG shells reduce the activation of the complement systems and prolong vascular circulation of liposomes (Salmaso and Caliceti 2013). Aiming for good tolerability but also for superior loadability of dendrimers, *core-multishell (CMS) nanotransporters* have been developed. These are surface modified dendrimers, the dendrimer core being covalently linked to a lipophilic inner shell and an outer PEG shell (Fig. 15.1). Due to the specific structure combining lipophilic and more hydrophilic regions, CMS nanotransporters are able to transport a broad spectrum of agents. Besides parenteral application, CMS nanotransporters are an option for topical drug use. The current research in CMS nanoparticles aims for the topical drug application for the treatment of different skin diseases. Related particles are dendritic hyperbranched polyglycerol-only nanoparticles (Khandare et al. 2012; Quadir and Haag 2012) and biodegradable dendritic nanogels. These are nanosized networks of chemically or physically cross-linked polymer nanoparticles, e.g., from PG (Steinhilber et al. 2013) or polyacrylic acids and polyacrylamides 100–1000 nm in diameter (Samah et al. 2010). Another related system is the *tecto-dendrimer,* a medium-sized dendritic core that is covered by a shell of small dendrimers (Schilrreff et al. 2012).

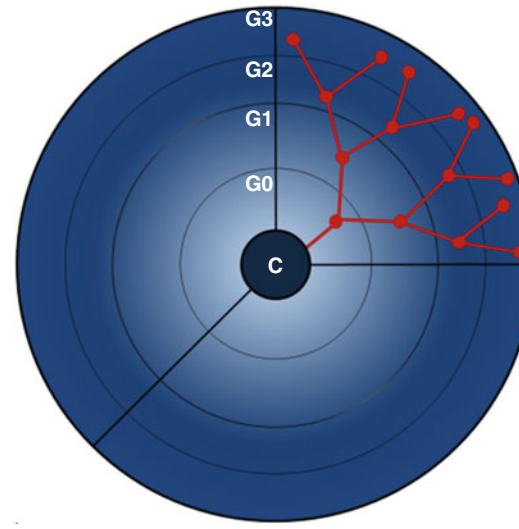

Fig. 15.1 Drug-loaded dendrimer (*C* core, *G* generations; modified from (Sun et al. 2012))

15.2 Physical and Chemical Properties of Core-Shell Nanotransporters

Dendrimers are small-sized, synthetic, defined, hyperbranched macromolecules with a tree-like architecture (dendritic polymers; Fig. 15.1) which have been discovered about 40 years ago. Starting from a central multivalent small (initiator core scaffold) molecule, repeating branching units are attached by covalent linkages resulting in nanoparticles of 1 to about 10 nm in size. Polyamidoamine (PAMAM), poly(L-lysine), but also polypropylenimine (PPI), polyethylenimine (PEI), and polyglycerol (PG) scaffold dendrimers are commonly referenced (Mignani et al. 2013). The size results from the numbers of repeated synthetic steps, the "generations" (G) of the dendrimers. With higher generations, the nanoparticles are spherically shaped. Size and shape as well as remaining functional groups can be precisely manipulated. Following intravenous administration, dendrimers strongly escape from the uptake by the reticuloendothelial system (RES) and thus circulate in the blood for long.

Drugs can be embedded into cavities of the nanoparticle. In general the generations G4 to G6 provide suitable cavities for stable drug loading by supramolecular interactions, whereas the void spaces are often too narrow with smaller dendrimers (up to G3) and too wide and thus leaky with G7 or larger dendrimers. Alternatively, drugs can be linked covalently to the multiple, terminally active functional groups (e.g., –OH, $–NH_2–$, COOH).

In addition proteins can be linked for active targeting of the dendrimer. Modified dendrimers, in particular modifications of the particle surface, have been synthesized and tested in order to improve loadability, stability of drug loading in the different buffers and plasma, and tolerability. Surface modifications result in structures built from a dendritic core covered by one or more shells. pH-responsive nanoparticles are another aim for improved drug delivery. Controlled release of an encapsulated guest molecule becomes possible by, e.g., acid-sensitive linkages included into the carrier shell. Approximately 30 % shell cleavage destroyed the ability of the nanoparticle to retain the agent (Krämer et al. 2002).

Inspired by the structure of liposomes and aiming for the generation of nanoparticles that are compatible with various environments, in particular the blood, the dendrimer technology has been taken up for the development of the complex *CMS nanotransporters*. These are built by hyperbranched dendritic cores which are surrounded by *double-layered shells* (Fig. 15.2). Initially, a PEI core was used; later on the cores were of PG, too (Küchler et al. 2009b), because of safety reasons. Cationic nanoparticles including the dendrimers are in general more cytotoxic than neutral ones (Venuganti et al. 2011; Wu et al. 2010). For synthesis of CMS nanotransporters, functional (–OH, $–NH_2$) groups of the core surface are covalently linked to a dicarboxylic

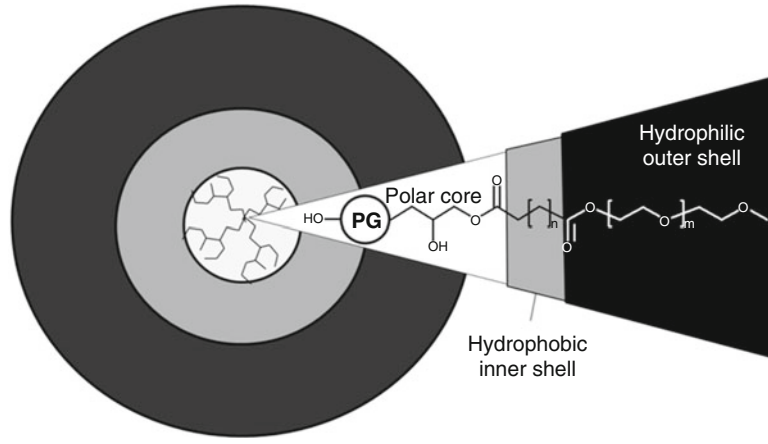

Fig. 15.2 Schematic representation of dendritic core-multishell nanotransporters (Modified from (Radowski et al. 2007))

acid (e.g., C_6, C_{12}, C_{18}), and monomethyl poly(ethylene glycol) (mPEG with 6, 10, or 14 glycol units) is connected to the peripheral free carboxylic acid in order to bind the outer shell to the inner shell (Radowski et al. 2007). CMS nanotransporters well dissolve in water and organic solvents. The individual units are about 8–9 nm in diameter, yet above a rather low concentration, CMS nanotransporters aggregate forming clusters which are about 20–30 nm size (Fig. 15.1). The nonpolar inner shell can incorporate lipophilic agents, while the core and outer PEG shell can incorporate hydrophilic compounds. Subsequently, CMS nanotransporters incorporate lipophilic agents, such as β-carotene and the fluorescent probe Nile red, as well as hydrophilic agents, e.g., the dyes Congo red and Rhodamine B (Küchler et al. 2009a).

Besides CMS nanotransporters, structure-controlled or *core-shell* tecto-dendrimers are supra-macromolecular new polymers that lack the structural drawbacks of dendrimers. Similar in size to G6–G8 dendrimers, the *core-shell* tecto-dendrimers can be synthesized in less steps and the *de Gennes* dense-packing phenomenon does not occur. With conventional dendrimers of G above 5, the polymer surface becomes crowded with terminal functional groups (*de Gennes* phenomenon) that shield the access of foreign guest molecules to the inner pockets of the dendrimer. Since this rigid and closed structure is no longer a unimolecular micelle (with drug dissolved within the dendrimer structure), high G dendrimers are not useful as drug carriers.

The *core-shell* tecto-dendrimers are built from dendrimer units only. Tecto-dendrimers consist of a core made of a single cationic dendrimer, covalently linked to a shell of small anionic dendrimers (Li et al. 1999; Tomalia et al. 2000; Uppuluri et al. 2000). For example, by combining an amine-terminated core dendrimer with an excess of carboxylic acid-terminated dendrimers, a saturated shell of dendrimers parked around is obtained. Then almost 100 % of the available positions in the dendrimer core have reacted (Uppuluri et al. 2000). To meet that point, first the cationic cores and anionic shells are allowed to equilibrate and self-assemble into the electrostatically driven core-shell tecto-dendrimer architecture. Then core and shell are covalently fixed with carbodiimide reagent. As an example, starting from PAMAM G5 as a core and a PAMAM G3-carboxylic acid terminated as shell, a core-shell tecto-dendrimer (G5 (core) G3 (shell) = G5G3) can be obtained in a single step at a high yield (i.e., 90 %). The size is between that of a G7 and a G8 dendrimer and the saturation degree 75–83 % (Uppuluri et al. 2000).

15.3 Delivery of Agents Loaded in CMS Nanotransporters to the Skin

15.3.1 Interaction with Keratinocytes

CMS nanotransporters and solid lipid nanoparticles (SLN; aggregated lipids solid at room temperature) are rapidly taken up by primary human keratinocytes grown in monolayers. Uptake is reduced but not excluded at low temperature and by energy depletion; both energy-independent diffusion and energy-dependent process are involved (Küchler et al. 2009b). This is well in accordance with the mechanisms of internalization of CMS nanotransporters by cancer cells – diffusion and receptor-mediated endocytosis, respectively. Uptake is also close to internalization of liposomes and of dendritic polyglycerols (Khandare et al. 2012; Quadir and Haag 2012).

Thus, CMS nanotransporters can efficiently deliver their payload to viable keratinocytes and potentially also to fibroblasts, if the particles surmount the penetration barrier. This makes them interesting carriers for nonmelanoma skin cancers which develops from transformed keratinocytes. Melanoma may be also an indication for the use of CMS nanotransporters.

15.3.2 Penetration Enhancement by CMS Nanotransporters: Normal Skin

Human skin clearly differs from the skin of rodents in the density of hair follicles. Being 0.1–1 % of the surface in human skin, hair follicles are much more abundant in laboratory

animals, such as mice and rats. Consequently, studies in rodents are strongly over-predictive for the penetration of actives into and permeation through human skin. Pig skin, however, is an accepted test matrix for evaluating skin penetration of actives (Diembeck et al. 1999). This holds also true with reconstructed human epidermis, despite of a weaker barrier function (Schäfer-Korting et al. 2008) due to a less-ordered organization of stratum corneum lipids (Van Gele et al. 2011). The barrier in human skin is formed by corneocytes and the intercellular lipids composed of ceramides, cholesterol, cholesterol sulfate, and free fatty acids being profoundly organized in the stratum corneum (Janusova et al. 2011). An orthorhombic structure (organization) is often found (Bouwstra and Ponec 2006). Waxes and squalene from the sebum can add to the skin barrier function. Tight junctions found in the stratum granulosum and lower stratum corneum appear less relevant with respect to the skin barrier function and penetration of actives (Brandner et al. 2006).

Loading lipophilic and hydrophilic probes into CMS nanotransporters which form aggregates of 20–30 nm clearly enhances skin delivery of the agents as observed in several studies in human and pig skin ex vivo. The penetration of Nile red into the viable epidermis of human skin was enhanced about 13-fold by CMS nanotransporters, whereas SLN enhanced the uptake about sixfold compared to a hydrophilic cream. Penetration into the dermis increased, too, yet to a lower extent (Küchler et al. 2009b). A previous study comparing Nile red penetration into pig skin from different carriers proved SLN to be the most efficient lipid nanocarrier. The penetration enhancement was the following order: SLN > NLC (nanostructured lipid carriers built by mixtures of lipids solid and fluid at room temperature) > cream = nanoemulsions (Lombardi Borgia et al. 2005). An improved penetration of the lipophilic spin probe TEMPO into pig skin ex vivo and skin of the forearm of human volunteers was obtained with NLC and invasomes, respectively. Yet, this was in particular due to the stabilization of the reactive nitroxide label by lipids in the carrier and the skin (Haag et al. 2011a, c). SLN-related enhancement of skin penetration was also obtained with glucocorticoids (Santos Maia et al. 2002; Schlupp et al. 2011) possessing lipophilicity close to that of Nile red. This proves the suitability of the dye probe for the estimation of penetration of lipophilic agents in general as well as the relevance of data generated with the Nile red-loaded CMS nanotransporters (Fig. 15.3).

CMS nanotransporters and SLN proved also more efficient in skin delivery of the hydrophilic probe Rhodamine B when compared to a cream. CMS nanotransporters and SLN-enhanced dye levels were 11.5-fold and ninefold, respectively, in the viable epidermis of pig skin ex vivo. In the same study, we investigated liquid nanocapsules; these are neutral oil shells enclosing an aqueous solution of Rhodamine B. Results indicated no differences in skin penetration with nanoparticles varying in sizes of 60–185 nm (Küchler et al. 2009a). Penetration of the hydrophilic spin probe 3-carboxy-2,2,5,5-tetramethyl-1-pyrrolidinyloxy (PCA) into the stratum corneum of porcine skin ex vivo was enhanced 2.5-fold by CMS nanotransporters and 1.9-fold by invasomes. PCA is exclusively localized in the hydrophilic compartments of CMS nanotransporter solutions and invasome dispersions (Haag et al. 2011b).

Based on the aforementioned results, it is obvious that CMS nanotransporters were superior to other nanocarriers in terms of enhancing actives' penetration into the skin. Yet, the precise delivery mode of guest molecules by CMS nanotransporters still needs to be unraveled. Non-surface-covered dendrimers and SLN, however, have been studied in more detail and first proposals encompass that both may act as drug-release modifiers and facilitate drug transfer from the vehicle/nanoparticle to the skin lipids which often is rate-limiting in skin penetration of drugs. In a systematic comparison, dendrimers (G4-NH2, G3.5-COOH, G4-OH about 15 nm in size; covalent FITC label) were mainly limited to the intact stratum corneum of porcine skin, the cationic dendrimers most efficiently penetrated into the stratum corneum. Penetration of a cationic G2 dendrimer exceeded the penetration of the G4 dendrimer, whereas a poorer penetration was

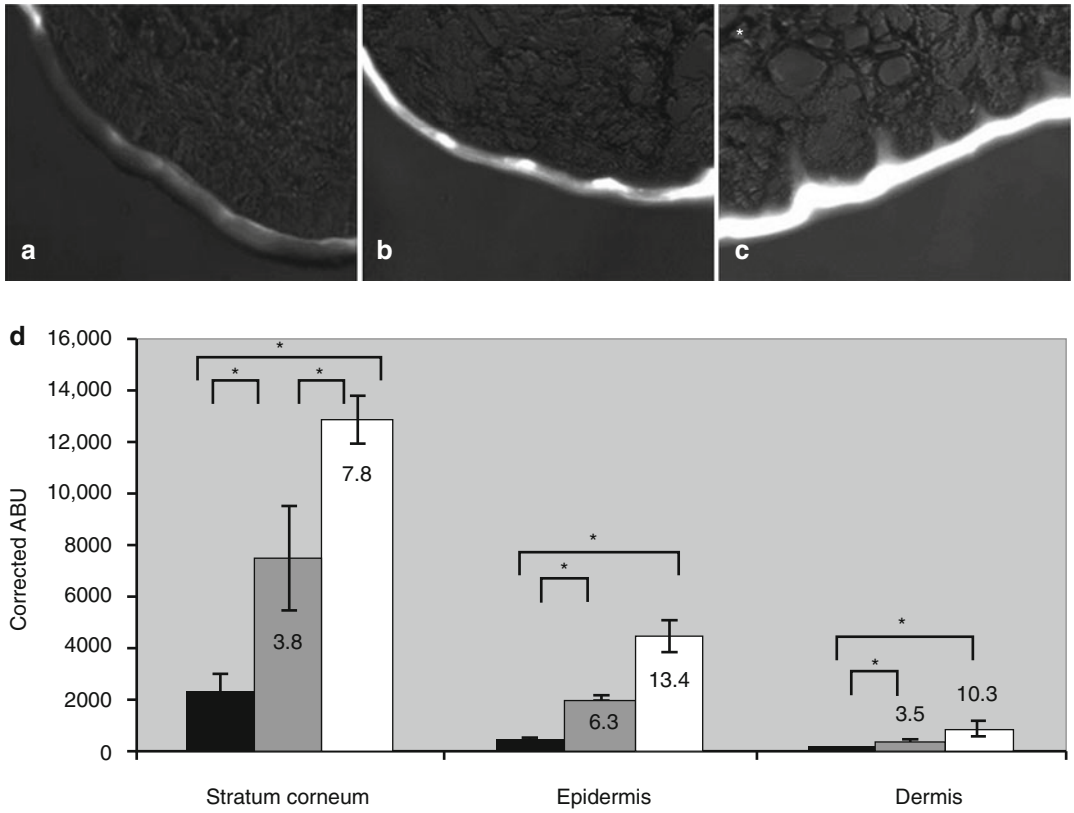

Fig. 15.3 Penetration of Nile red into human skin ex vivo with CMS nanotransporters and SLN. Overlays of morphology in fluorescence of nile read loaded to (**a**) hydrophilic cream, (**b**) solid lipid nanoparticles, (**c**) CMS nanotransporters, (**d**) Nile red concentrations as derived by picture analysis (arbitrary fluorescence units, ABU, corected for background fluorescence), *p<0.05 (From (Küchler et al. 2009b); with permission)

seen with the respective G6 dendrimer (Venuganti et al. 2011). This proves the relevance of an electrostatic interaction with the acid skin surface observed previously when studying polymer particles approximately 100 nm in diameter (Wu et al. 2010) and the anticipated size effect. Minor amounts were detected in viable epidermis which could be enhanced by iontophoresis (Venuganti et al. 2011). Yet the authors failed to prove the stability of the label; esterases are found in high amounts in viable skin and on the skin surface (Bätz et al. 2013). Moreover, access to viable skin may result from penetration via shunt pathways of porcine skin. Following topical application, *particle adhesion* to the stratum corneum and interaction of the nanoparticles with *skin surface lipids* are the basis of enhanced delivery of lipophilic agents to the skin by SLN (Küchler et al. 2009b; Kuntsche et al. 2008). In fact, SLN most efficiently deliver spin probes to sebum and stratum corneum lipids (Küchler et al. 2010). SLN made from the highly lipophilic lipid glycerol behenate do not interfere with synthetic phospholipid membranes either (Blaschke et al. 2010). Other than the SLN, cubic nanoparticles also interfere with *stratum corneum lipids* by the penetration of the polar lipid glycerol monooleate into the stratum corneum and thus disturb the barrier (Kuntsche et al. 2008).

Moreover, low-generation dendrimers, in particular cationic particles, may impair the stratum corneum barrier by fluidization of the lipid bilayers of the stratum corneum. Penetration of dendrimers into hair follicles and delivery of their guest molecules to the hair follicle infundibulum or the sebum gland (Mignani et al. 2013; Sun et al. 2012) should not be relevant with the very small dendrimers as the optimum particle

size of hair follicle targeting is around 600 nm (Lademann et al. 2008; Patzelt et al. 2011).

15.3.3 Penetration Enhancement by CMS Nanotransporters: Diseased Skin

Currently, the influence of skin diseases on nanoparticle-enhanced drug penetration is almost unknown. However, it is known that in skin diseases major changes in the stratum corneum barrier occur, e.g., in atopic skin a hexagonal orientation of the stratum corneum lipids is more prominent while orthorhombic structures decline (Pilgram et al, 2001). The relevance of the impaired barrier function in skin diseases for skin absorption of drugs is discussed controversially (Groen et al. 2011). The very limited availability of lesional skin for experimental purposes almost excludes investigations of skin penetration enhancement by nanoparticles. An alternative is the use of reconstructed human diseased skin which has been described recently (Semlin et al. 2011). Pathophysiologically relevant defects can be induced by gene silencing in normal keratinocytes which are then used for tissue reconstruction. For the model of peeling skin disease, the *corneodesmosin* gene is knocked down (Oji et al. 2010). Silencing the *filaggrin* gene is an approach to other skin diseases with barrier defects, e.g., psoriasis and atopic dermatitis (Mildner et al. 2010; Küchler et al. 2011).

Impairing the barrier function by filaggrin knockdown results in enhanced levels of free fatty acids and a less ordered structure of stratum corneum lipids (Vávrová et al. 2014). The skin model is super permeable for the lipophilic standard compound testosterone but not for the hydrophilic standard compound caffeine (Küchler et al. 2011). In contrast, caffeine and testosterone permeate more efficiently constructs reflecting peeling skin disease than reconstructed normal skin (Oji et al. 2010). These results strongly indicate the need for investigations on skin penetration of drugs from drug formulations (including nanoparticles) in the respective skin disease models.

Surmounting the impaired skin barrier by intact CMS nanotransporters can result in a significant enhancement of intracellular drug levels due to the internalization of the nanoparticles by keratinocytes (Küchler et al. 2009a) and release of loaded drug, e.g., in lysosomes or cytoplasm (Quadir and Haag 2012). Interpretation of the results derived from studies using reconstructed human skin indicating the penetration of intact nanoparticles, however, has to be done with caution since the composition and organization of stratum corneum lipids in the constructs differ from that in the human skin (Janusova et al. 2011). A difference seen in animal and human skin was attributed to differences in stratum corneum lipids (Kuntsche et al. 2008).

15.4 Safety Considerations

CMS nanotransporters in particular those with a polyglycerol core are devoid of variables known to be not infrequently associated with poorer tolerability (PEI core, cationic surface, high surface reactivity; (Khandare et al. 2012)). The CMS nanotransporter which is proven to be very efficient with respect to Nile red delivery to human skin has been subjected to standardized testing for local adverse effects, too. This nanoparticle did not induce a major (≥30 %) decline in the viability of primary human keratinocytes following 24 h exposure, and toxicity was not seen in standardized in vitro testing for irritation of the skin (EpiSkin Test; OECD 432) or the eye (HET-CAM test) either (Küchler et al. 2009b). Moreover, also a nonanoyl-functionalized PEG-covered dendritic polyglycerol nanoparticle did not induce cytotoxicity in keratinocytes. This particle was obtained by the chemo-enzymatic modification of dendritic hyperbranched polyglycerol in order to improve Nile red encapsulation and for a pH-dependent drug release in inflamed areas (Kumar et al. 2011).

15.5 Proposed Clinical Use

For a congenital ichthyosis linked to deficient transglutaminase, substitution of the enzyme may relief the symptoms. Topical treatment is hampered by the disease-associated hyperkeratotic skin yet may be overcome by enzyme

encapsulation into nanoparticles. In fact, full enzyme activity after release has been obtained by loading the enzyme to cross-linked dendritic polyglycerol nanoparticles 11–1000 nm in size (Steinhilber et al. 2013). The nanoparticles described here may also offer a new treatment option for this and other in general recalcitrant skin diseases.

Moreover, CMS nanotransporters may allow an improvement in the therapy of nonmelanoma skin cancers by efficient delivery of anticancer nucleotides (Höltje et al. 2010; Zdrazil et al. 2010) and small-interface RNA (siRNA) delivery (Malhotra et al. 2012). Nonmelanoma skin cancer lesions are often hyperkeratotic, and thus access of topical applied drugs is impaired. SLN failed to enhance antitumor effects of the nucleoside OxBu (Ali-von Laue et al. 2014) in the respective tumor model (Hoeller Obrigkeit et al. 2009). The failure of SLN to enhance the activity of an anticancer nucleotide may be due to the antiapoptotic effect of glycerol behenate forming the SLN (Wolf et al. 2009).

New treatment options may be also achievable due to differences in penetration enhancement. CMS nanotransporters appear to favor the epidermal penetration of Nile red over the dermal penetration (Küchler et al. 2009b) as shown also for SLN (Chen et al. 2006; Santos Maia et al. 2002; Schlupp et al. 2011; Stecova et al. 2007). If future studies will prove this observation, CMS nanotransporters and SLN may gain specific interest in the therapy of atopic dermatitis because of epidermal targeting. Whereas symptoms improve predominantly due to the suppression of the epidermal inflammation, glucocorticoid-induced skin atrophy results from reduced collagen amounts in the dermis (Weindl et al. 2011). A decline in the most relevant adverse effect of topical glucocorticoids may allow a less restricted prescription of these highly active drugs. Current research in the field of topical therapy focusses on enhanced drug delivery. As outlined above, drug targeting to specific skin strata seems possible to, at least, a limited extent. Following intravenous administration, targeting is possible, too. This includes passive process due to size-related enrichment in tumors and inflamed areas due to the leaky blood vessels and a less efficient draining by lymphatic vessels in these areas, which is called EPR (enhanced permeability retention) effect. Active targeting becomes possible by covalent binding of, e.g., tumor-specific ligands to terminally active functional groups of the dendrimers.

Interestingly, dendrimers can act as drugs, too. In 2012 Starpharma (Melbourne, Australia) has entered into phase III clinical testing of a microbicide, anionic G4-poly-(L-lysine) dendrimer (Vivagel®) with attached naphthalene disulfonate groups to its surface for vaginal infections (Mignani et al. 2013). Moreover, Rainer Haag's group has demonstrated strong anti-inflammatory potency of dendritic polyglycerol sulfates, inhibiting leukocytic P-selectin and endothelial E-selectin, efficacy being close to prednisolone in a murine model of contact dermatitis (Dernedde et al. 2010).

Although the synthesis and structural characterization of tecto-dendrimers were described nearly 13 years ago, the interaction between tecto-dendrimers and mammal cells remained unexplored until recently (Schilrreff et al. 2012). In particular, the cytotoxicity of G5G2.5 *core-shell* tecto-dendrimers (made of a cationic-PAMAM dendrimer G5 (MW: 28826, 128 amine surface groups) as a core and anionic-PAMAM dendrimers G2.5 (MW: 6265, 32 carboxylic surface groups as shell) on Caco-2, HaCaT, and SK-Mel-28 cells is similar to that of G2.5 anionic dendrimers and considerably lower than that of G5 cationic dendrimer. However, SK-Mel-28 cells are capable of sensing subtle structural differences such as those between core-shell tecto-dendrimers and dendrimers of similar MW and size but higher surface congestion. This was observed for a commercial anionic PAMAM dendrimer G6.5 close in structure to the G5G2.5 tecto-dendrimer in terms of chemical structure, size, and MW but with twofold higher surface congestion. Most interestingly, non-loaded G5G2.5 tecto-dendrimer is selectively cytotoxic to melanoma cells while G6.5 is not.

G5G2.5 is avidly taken up by the melanoma cell line SK-Mel-28. The clearly less efficient

uptake by the keratinocyte-derived (HaCaT) and enterocyte-derived (Caco-2) cell lines may allow for selective effects on tumor cells. Importantly, the G5G2.5 dendrimer displays a selective and concentration-dependent activity on SK-Mel-28 cells (IC_{50} 7.5 µM), in the absence of cytotoxicity for HaCaT and Caco-2 cells. The G5G2.5 cytotoxicity is related to a rapid depletion of intracellular glutathione (GSH). Melanoma cells are particularly sensitive for oxidative stress (Farmer et al. 2003; Meyskens et al. 1999, 2001; Schilrreff et al. 2012) which may be attributed to constitutive abnormalities of the tumor cell. The intracellular GSH level of SK-Mel-28 cells is ~80–100 nmol/mg protein and 7300 nmol/mg protein in HaCaT cells (Snow et al. 2005).

Moreover, SK-Mel-28 cells are in particular sensitive to MTX loaded to G5G2.5 (Schilrreff et al. submitted) which is not true with unloaded MTX. As a ligand of the folate α receptor, free MTX is internalized by melanoma cells. Yet, taken up by melanosomes, MTX traffics a pathway that leads to exocytosis of the antitumor drug (Sanchez-del-Campo et al. 2009). Thus efficient cytosolic accumulation of MTX and its subsequent inhibition of the enzyme dihydrofolate reductase are excluded. This does not hold true with G6G2.5-loaded MTX. Melanoma cells tolerate up to 100 µM free MTX. However, 50 µM MTX loaded to the tecto-dendrimer is toxic for melanoma cells, while HaCaT and Caco-2 cells remain unaffected. This documents the potential of the tecto-dendrimers for the treatment of skin diseases.

15.6 Concluding Remarks

Taken together all available results, CMS nanotransporters and related nanoparticles influence drug penetration into the skin. Moreover, specific dendrimers including tecto-dendrimers appear even to act as drugs by themselves. Given toxicological studies will demonstrate sufficient tolerability of the agents, these nanoparticles may improve topical therapy of skin diseases and therefore should be tested in vivo, too.

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Microemulsions

16

Sari Pappinen and Arto Urtti

Contents

16.1 Introduction

The structure of microemulsion was first introduced by Hoar and Schulman in 1943, when conventional emulsion structure was transformed to transparent solution after the addition of co-surfactant into the system. Ever since, microemulsions have been used in several fields of industry. Potential of thermodynamically stable and low-viscosity microemulsion systems in drug delivery has also been recognized in pharmaceutical industry, and the microemulsions have gained a lot of interest in cutaneous drug delivery during the past few decades. The first articles about the potential use of microemulsions as topical formulations were published in the 1980s (Martini et al. 1984; Wang et al. 1987).

Microemulsions are formed spontaneously when oil, water, and fixed mixture of surfactant/co-surfactant are combined. They are effective vehicles in drug delivery due to their facile and low-cost preparation, high solubilization capacity of various drugs, increased bioavailability, and thermodynamic stability (Fig. 16.1). Typical transparency and low viscosity of microemulsions are due to their small droplet diameter that enables high mobility in the vehicle and adhesion to the skin surface and thereby enhance drug permeation. It seems that microemulsions are able to mediate higher percutaneous drug delivery compared to emulsions, hydrogels, and liposomes (Kriwet and Muller-Goymann 1995; Paolino et al. 2002) (Fig. 16.1).

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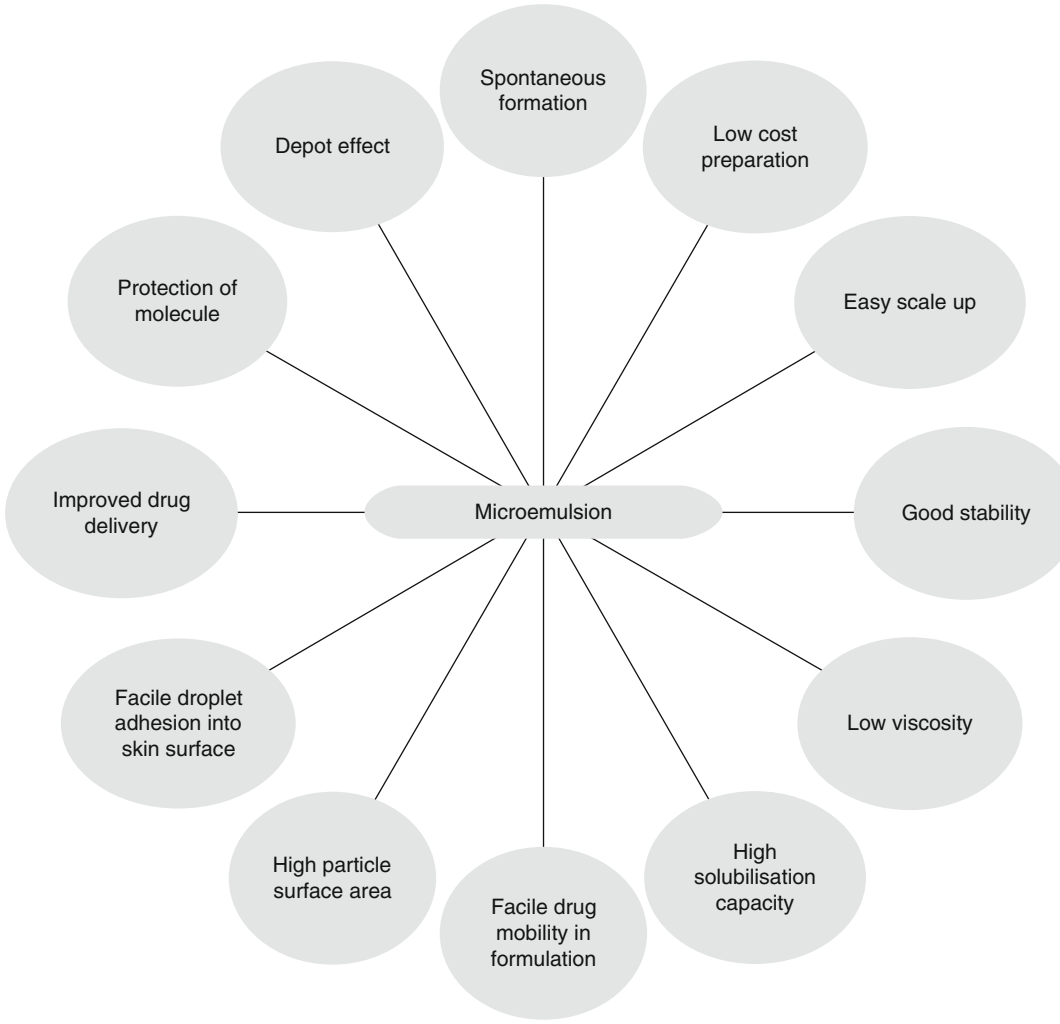

Fig. 16.1 Characteristic features of microemulsions

16.2 Microemulsion Structure and Composition

Microemulsions are formed spontaneously when oil, water, and a fixed mixture of surfactant/co-surfactant are combined (Table 16.1). The oil and water components of microemulsion can be either in a continuous external phase or enclosed into the dispersed phase (o/w or w/o microemulsions), when the surfactant and co-surfactant have a strong tendency of adsorption at the phase interfaces (Kreilgaard et al. 2000; Shukla et al. 2003) (Fig. 16.2). Microemulsion structure is obtained using a range of oil–water–surfactant compositions, but in the majority of cases, the microemulsions exist only over a narrow range of concentrations. Selecting the right amount of each excipient in the formulation requires construction of pseudoternary phase diagrams, in which each corner of the diagram represents 100 % of the particular component or binary mixture (Fig. 16.3). A simple change in the relevant percentage of microemulsion components allows the formation of other colloidal structures such as regular emulsion, anisotropic crystalline hexagonal or cubic phases, and lamellar structures (Baroli et al. 2000).

Table 16.1 Components in topical microemulsions

Oil phase	Surfactant	Co-surfactant	Aqueous phase
Saturated and unsaturated fatty acids e.g., oleic acid, linoleic acid	*Natural surfactants* e.g., lecithin, alkyl glucosides, akylesters	*Short- and medium-chain alcohols* e.g., ethanol, 1-butanol, decanol	Water Buffer solution
Fatty acid esters e.g., isopropyl myristate, isopropyl palmitate	*Nonionic surfactants* e.g., Plurol Isostearique, polysorbates (Tween 20, Tween 80), sorbitane monooleate (Span 80), polyethylene glycol lauryl ether (Brij 35), poloxamer	*Other* Transcutol Propylene glycol	
Triglycerides e.g., caprylic/capric acid triglycerides, triacetin, isostearylic isostearate, soybean oil, olive oil	*Other* Labrasol		

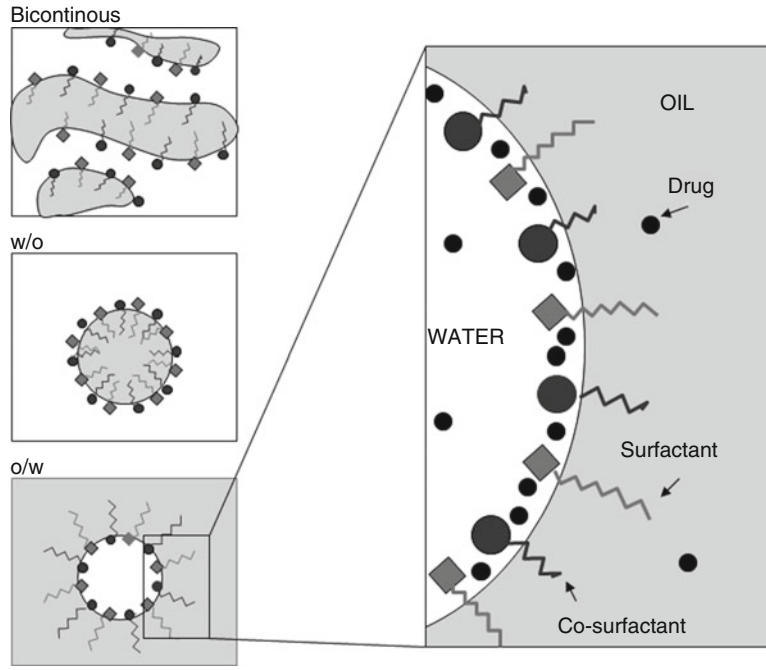

Fig. 16.2 The basic microemulsion structure formed by oil, water, surfactant, and co-surfactant interfacial film (From Pappinen and Urtti (2006): 109. With permission)

Microemulsions are spontaneously formed when the interfacial tension at the oil/water interface is brought to a very low level and the interfacial layer is kept highly flexible and fluid. This typically requires the use of co-surfactant, e.g., short-chain alcohols, in combination with surfactant, but also careful choice of the other microemulsion components and their proportions to provide flexibility to the oil/water interface. Furthermore, various oils in the microemulsions have influence on the flexibility of interfaces (Trotta et al. 1997; Alany et al. 2000; Malcolmson et al. 1998). It has been suggested that small molecular volume oils, such as saturated and unsaturated fatty acids, would be located in the interfacial surfactant monolayer (like a surfactant), while larger oil molecules, such as triglycerides, tend to be located in the center of the droplet. The low surface tension leads to subdivision of the dispersed phase into very small

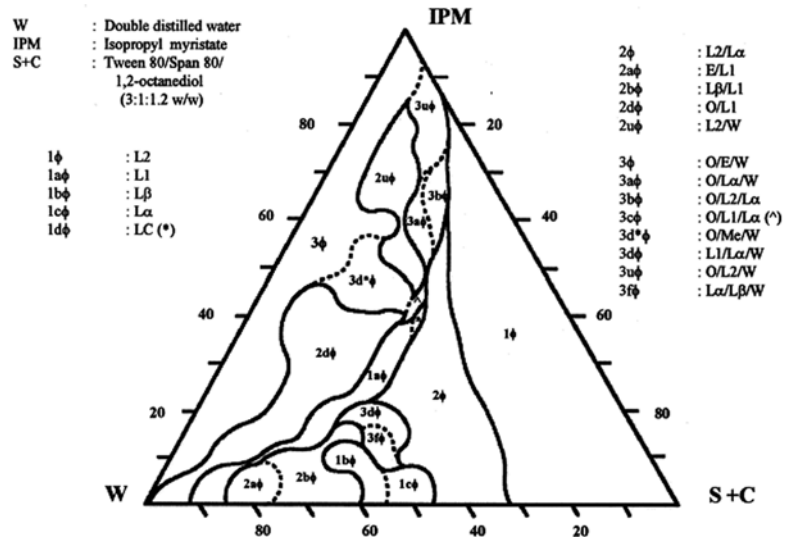

Fig. 16.3 Pseudoternary diagram of the W:IPM:S1C system at 25 °C. The diagram is characterized by the presence of regions of one, two, or three phases (1f, 2f, 3f). L2 and L1 phases delimit the regions of W/O and O/W microemulsions, respectively. La and Lb correspond to lamellar liquid crystals and lamellar gel phases. LC phase is an anisotropic region of liquid crystals. In the case of the regions with several phases, Me stands for a bicontinuous microemulsion and E for emulsion (From Baroli et al. (2000); 69:209. With permission)

droplets, an increase in the number of droplets, and enlargement in the total internal surface area of oil/water interfaces (Kreilgaard et al. 2000; Lam and Schechter 1987). Moreover, low surface tension ensures good contact with the skin.

Microemulsions are very different from coarse emulsions that require high-energy input in formation and are cloudy in appearance and less stable than microemulsions. In contrast to emulsions, microemulsions contain much more surfactant and, unlike emulsions, also co-surfactant that enable very low interfacial energy. Therefore the main difference between emulsions and microemulsions lies in the size of the droplets dispersed in the continuous phase that are at least an order of magnitude smaller in the case of microemulsions (10–100 nm) than those of conventional emulsions (1–20 μm). Transparency and low viscosity of microemulsions arise from their small droplet diameter (Tenjarla 1999). Unlike coarse emulsions microemulsions are fluctuating systems in which the droplets are aggregated due to the very flexible interfacial film; thereafter they are again separated to smaller droplets (Kreilgaard et al. 2000; Lam and Schechter 1987; Lee et al. 2003). Although there are continuous diffusional processes and collisions at the interfaces, the equilibrium size and shape of the droplet is maintained. This kind of dynamic system facilitates drug diffusion and release from formulation.

Characterization of microemulsion structure is challenging due to the wide variety of structures and kinetic processes, and combination of more than one technology is required for a reliable characterization. Many technologies are introduced to characterize microemulsion structures, such as dynamic light scattering (DLS) (Porras et al. 2004), small-angle neutron scattering (SANS) (Burnett et al. 2004), small-angle X-ray scattering (SAXS) (Podlogar et al. 2004), cryotransmission electron microscopy (Danino et al. 2002), and nuclear magnetic resonance spectroscopy (NMR) (Kreilgaard et al. 2000).

16.3 Means of Increasing Dermal Drug Delivery

16.3.1 Solubilization Capacity

It has been observed in several studies that a large amount of drugs can be incorporated to microemulsions due to their good solubilization capacity. Microemulsion components, like oil phase and surfactant/co-surfactant, have different high solubilizing potentials. The structure of microemulsion may contain even equal amounts of oil and water phases and a high surfactant content (20–80 %), enabling incorporation of large fractions of both lipophilic and hydrophilic drugs into the for-

mulation (Kriwet and Muller-Goymann 1995; Baroli et al. 2000; Trotta et al. 1997; Lee et al. 2003; Rhee et al. 2001; Alvarez-Figueroa and Blanco-MËndez 2001; Ktistis and Niopas 1998).

High solubilization potential of microemulsion allows an increase in the concentration gradient across the skin, which facilitate drug permeation according to Fick's law for diffusion (Higuchi and Higuchi 1960). It should be, however, noted that the thermodynamic activity of the drug is the real driving force in drug release from the vehicle into the skin. The thermodynamic activity for drug molecule can be described as the ratio between the real concentration and the saturated concentration in microemulsion (Barry 1989). That is, the maximal thermodynamic activity can be achieved when there is the saturated microemulsion.

In microemulsions the drug is assumed to be in equilibrium between the dispersed and continuous phase (Mrestani et al. 1998). Mostly drugs are associated with the interfacial surfactant film between inner and outer phase (Paolino et al. 2002; Kreilgaard et al. 2000; Sintov and Shapiro 2004; Chen et al. 2004) (Fig. 16.2). The role of the microemulsion structure in solubilization is not expected to be important, and solubility of the drug in microemulsions depends mostly on its solubility in the neat vehicle components (Peltola et al. 2003; Subramanian et al. 2005).

16.3.2 Drug Release from the Vehicle

Microemulsions are known to be dynamic systems of continuously colliding, disappearing, and newly forming droplets, which are affected by the flexibility of interfacial film and thus promoting high drug diffusion in the formulation (Kreilgaard et al. 2000; Lam and Schechter 1987; Lee et al. 2003). Release of a drug from a vehicle is affected by its tendency to stay in the microemulsion components or disperse structures and its tendency to partition from the vehicle into the skin. This is mostly affected by the physicochemical properties, like lipophilicity and solubility, of drug molecule but also by microemulsion structure. The random movement of a molecule in an isotropic solution without thermal gradients is called self-diffusion. Increased self-diffusion of drug in microemulsions is inversely related to the lower viscosity of vehicle and the higher rate of permeation across the skin (Kreilgaard et al. 2000; Hua et al. 2004).

Microemulsions have been demonstrated to improve transdermal delivery of several drugs compared to the conventional formulations, such as emulsions, gels, liposomes, aqueous solutions, and neat oil phases (Kriwet and Muller-Goymann 1995; Paolino et al. 2002; Kreilgaard et al. 2000; Ktistis and Niopas 1998). Superiority of microemulsion to other colloidal structures and conventional formulations is most likely due to the higher mobility and lower viscosity that related to increased drug release from microemulsion vehicle (Elshafeey et al. 2009). Small droplets provide better adherence to membranes and deliver molecules into the skin in more controlled fashion. Transformation of microemulsion to other colloidal structures, like micelles and lamellar vesicles, may decrease drug delivery through the skin (Trotta 1999).

Overall, the drug release from microemulsions is a complex interplay between the properties of the drug, the vehicle, and the skin barrier.

16.3.3 Drug Distribution and Permeation Enhancement

The mechanism of drug delivery from the microemulsion into and through the skin is not totally understood, but the complexity of microemulsion structure means that several factors may be involved simultaneously (Fig. 16.4). Complex nature of microemulsion is well reflected by the perception that even very similar microemulsions, which are composed of different ratios of same components, deliver drug at various extents, which might be attributed to diverse viscosities and droplet size (Elshafeey et al. 2009). Relative contribution of different mechanisms to the penetration of the drug may depend on the properties of the drug and microemulsion formulation.

The drug is distributed between the dispersed and the continuous phase depending on its

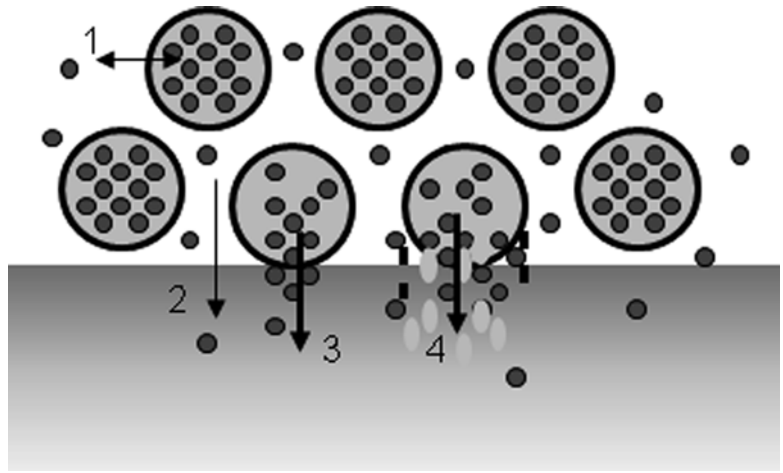

Fig. 16.4 Mechanisms of drug release from microemulsion droplets. Drug is always distributed between inner and outer phase according to its solubility to these components (*1*). In the first mechanism, drug penetrates to skin only from outer phase (*2*). In the second mechanism, high density of droplets in microemulsion enables the direct drug diffusion to the skin from a microemulsion droplet (*3*). In the third mechanism, droplets brake down upon contact with the skin and then release the contents of droplet into the skin (*4*). The microemulsion components may mix with skin lipids

lipophilicity and solubility, and it may be possible that drug partitions into the skin from the continuous phase (Fig. 16.4, 1–2). Subsequently, the absorbed drug is replaced by the drug released from the dispersed phase to the continuous phase, and the droplets function as drug reservoirs. Therefore the drug reservoir effect of the dispersed phase maintains an almost constant drug concentration gradient on the continuous phase and prolongs absorption (Peira et al. 2001).

The large surface area of microemulsion droplets in contact with the skin surface provides a high local concentration gradient that facilitates drug permeation directly from the droplet interface to the skin (Paolino et al. 2002; Kreilgaard et al. 2000; Sintov and Shapiro 2004; Chen et al. 2004) (Fig. 16.4, 3). In this case, the disintegration of the droplet structure is not needed. Many drugs, such as prilocaine, lidocaine, ketoprofen, and triptolide, locate in the droplet interface in a same manner as the surfactant and co-surfactant (Paolino et al. 2002; Kreilgaard et al. 2000; Sintov and Shapiro 2004; Chen et al. 2004).

Another possible mechanism is the droplet fusion on the skin surface (Fig. 16.4, 4). Since many components of the microemulsions are known to be permeation enhancers, like oleic acid, isopropyl myristate, lecithin, and polysorbate 80 (Tween80), they might interfere with SC lipids and improve drug permeation or increase drug solubility inside the skin (Yuan et al. 2010; Yutani et al. 2012). Hathout and coworkers (Hathout et al. 2010a, 2010b) reported that by formulating the oleic acid, polysorbate 20 (Tween20), diethylene glycol monoethyl ether (Transcutol), and water as microemulsion, the relative uptake of components into the skin increased compared to the neat excipients. They also concluded that the conformational order of stratum corneum lipids was proportional to the level of the respected formulation components presented in the skin. Microemulsion components may also have synergistic effect that facilitates drug delivery.

Drug permeation has been further improved by adding the small amount of stronger permeation enhancers, like terpenes and DMSO, into the microemulsion system (Chen et al. 2004; Gasco et al. 1991). Incorporation of 5 % limonene to microemulsion has been reported to increase ketoprofen permeation by fivefold (Rhee et al. 2001) and diclofenac permeation by sevenfold (Escribano et al. 2003) over microemulsion control.

16.4 Efficacy and Safety of Microemulsion-Based Formulation

The enhancement potential of microemulsions as drug delivery systems has been demonstrated in many *in vitro* permeation and deposition studies (Kriwet and Muller-Goymann 1995; Baroli et al. 2000; Trotta et al. 1997; Lee et al. 2003; Rhee et al. 2001; Alvarez-Figueroa and Blanco-MËndez 2001; Ktistis and Niopas 1998). Improved dermal delivery potential of microemulsion over conventional topical formulations can be obtained (Kriwet and Muller-Goymann 1995; Kreilgaard et al. 2000; Ktistis and Niopas 1998; Zhu et al. 2009). A similar trend is also seen in *in vivo* studies where microemulsion based drug formulations have shown good pharmacokinetics and efficacy in several dermatological disorders, like local inflammation (bacterial, fungal, or viral) and atopic dermatitis, and therapeutic conditions such as in delivery of local analgesics and anesthetics (Subramanian et al. 2005; Elshafeey et al. 2009; Soliman et al. 2010; Tsai et al. 2010; Hashem et al. 2011; Dalmora et al. 2001; Zabka and Benkova 1995; Lehmann et al. 2001; Kreilgaard et al. 2001; Bonina et al. 1995).

Investigators have found a significant increase in drug permeation of celecoxib through rabbit and rat skin from a microemulsion, compared to cream (tenfold) and gel (fivefold) formulations (Subramanian et al. 2005; Soliman et al. 2010). The therapeutic efficacy of the microemulsion was superior over the cream, i.e., a 3.5-fold higher anti-inflammatory activity of celecoxib was reported (Soliman et al. 2010). Escribano et al. reported an up to tenfold enhanced permeation of diclofenac through excised human skin when the microemulsion was applied compared to the cream formulation (Escribano et al. 2003). Anti-inflammatory activity was not only greater but also started earlier than that after the application of the cream. In another study Paolino et al. reported that the permeation rate of ketoprofen through human skin was twofold and fivefold higher from a microemulsion than from a gel and a cream, respectively (Paolino et al. 2002). Microemulsions were also found to increase the permeation rate of both lipophilic (lidocaine) and hydrophilic (prilocaine) model drugs. A fourfold enhanced transdermal flux of lidocaine from a microemulsion compared to the commercially available lidocaine cream (Xylocaine®) was reported (Kreilgaard et al. 2000).

One of the main challenges, after applying a topical microemulsion onto the skin, is that the formulation may change. Nonoccluded application of a thin layer of microemulsion onto the skin surface enables evaporation of some components, like alcohols, or permeation of components into the skin at different rates and extents. This may lead to disruption of the microemulsion structure before absorption, which might decrease drug permeation since the transformation of microemulsion to other colloidal structures is known to decrease drug delivery through the excised skin (Trotta 1999). This effect has not been reported to influence on drug delivery in *in vivo* studies, and microemulsions are considered to be good formulation candidates in clinical use (Subramanian et al. 2005; Elshafeey et al. 2009; Soliman et al. 2010; Tsai et al. 2010; Hashem et al. 2011; Dalmora et al. 2001; Zabka and Benkova 1995; Lehmann et al. 2001; Kreilgaard et al. 2001; Bonina et al. 1995).

Microemulsions have the potential to increase drug permeation into the skin, as well as through the skin to the systemic circulation. Microemulsion was, e.g., shown to enhanced accumulation of the antioxidant quercetin at the target site in the skin without increased drug delivery to the systemic circulation (Vicentini et al. 2008). In other cases systemic drug delivery has been successful with plain microemulsions, like with testosterone microemulsions (Hathout et al. 2010a, 2010b). Innovative microemulsion-based patch formulations have also been developed as shown by Shi et al. (2012) in the study of simultaneous delivery of huperzine A and ligustrazine for the treatment of Alzheimer disease.

Large quantities of a surfactant and a co-surfactant are required for microemulsion formulations, and this, in principle, might cause skin intolerance, like irritation and sensitization. Skin irritation can be minimized by a careful selection

of the microemulsion components. The emphasis is, therefore, on the use of compounds that are generally regarded as safe and are clinically accepted for human topical formulations when used at appropriate concentrations. Generally, nonionic surfactants are less toxic than ionic surfactants, therefore, being more suitable for the use in microemulsions (Kreilgaard et al. 2000; Delgado-Charro et al. 1997). To overcome the safety issues, the microemulsions are formulated without co-surfactants and still good penetration enhancement properties are maintained. However, these microemulsions are more easily destabilized by changes in composition and temperature (Garti et al. 2001). Synthetic surfactants (such as polysorbate 80 (Tween 80), polyethylene glycol lauryl ether (Brij 35)) are also successfully replaced by natural surfactants, like lecithin, sucrose esters, and alkylpolyglycosides, which are biodegradable and only mildly irritating (Schwarz et al. 2012).

During the last few years, the skin compatibility of microemulsions has been evaluated in several studies in human and animal skin *in vivo* (Soliman et al. 2010; Hashem et al. 2011; Vicentini et al. 2008; Schwarz et al. 2012; Kantarc et al. 2007; Xu et al. 2008). In most cases, no signs of skin toxicity, such as erythema, edema, histopathological changes, and increased TEWL and blood flow, were observed. Sometimes skin irritation was caused by an increased absorption of an irritating drug, like diclofenac, rather than by the components of the microemulsion (Escribano et al. 2003). However, irritation studies are typically performed only for short periods of time, from few days to few weeks, and there are still open safety questions related to the chronic use of microemulsions. Local and systemic toxicity should be further studied and more human *in vivo* experiments are needed to determine therapeutic risks and benefits in clinical use.

16.5 Stability

Spontaneous formation of the microemulsion structure, with specific ratio of oil, water, and surfactant/co-surfactant, makes them energetically and thermodynamically stable systems. Stability of microemulsions has not been widely studied, but promising long-term stability results were recently reported at ambient and accelerated conditions (Soliman et al. 2010; Tsai et al. 2010; Censi et al. 2012). Physical characteristics, like clarity, homogeneity, phase separation, particle size distribution, viscosity, and pH, are typical markers of microemulsion stability. Incompatibility of drug molecules with microemulsion components might cause chemical degradation of drug molecules during storage, but this is not typically related to physical characteristics of microemulsion. It has been reported in a limited number of studies that microemulsions might also protect drug molecules enclosed in microemulsion droplets from oxidative degradation and hydrolysis (Chen et al. 2004; Spiclin et al. 2001), but more studies are still needed to confirm that. In conclusion, both physical and chemical stability of the drug can be controlled by the proper selection of the components and by the evaluation of the optimal ratio of components in microemulsion formulations.

Conclusion

Microemulsion structure is currently well characterized, but the complex and dynamic process of drug delivery to the skin is not fully understood. Nevertheless, the main contributing factors are known. Firstly, improved drug permeation with microemulsions is partly due to drug solubilization that ensures increased drug concentration gradient. Secondly, the enhanced drug delivery is related to high mobility of the drug after microemulsion administration. Thirdly, microemulsion components may fuse into the stratum corneum and increase drug partitioning into the skin, acting as penetration enhancers.

Systematic *in vivo* data of microemulsion-mediated drug delivery to the skin are rather limited, but improved drug delivery compared to conventional topical formulations has been reported in several studies. Safety of microemulsions in long-term use is not known, but in short-term use they are safe. Selection of the microemulsion components is the key

factor that eventually controls the skin tolerability, formulation stability, drug permeability, and clinical effect.

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17 Percutaneous Penetration Enhancement Potential of Microemulsion-Based Organogels

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Contents

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17.1 Introduction

Transdermal delivery is an alternative pathway to oral delivery of drugs which are unstable in the gastrointestinal tract (GIT) environment or suffer from low absorption, hepatic metabolism, short half-life, GIT side effects, or toxicity issues. Another area of great interest is a delivery of active ingredients into the deeper skin layers (dermal delivery). Despite the potential of dermal and transdermal delivery concept, the skin is an effective barrier for penetration of substances applying *via* conventional topical formulations (i.e., ointments, creams, hydrogels) (Barry 2002; Bronaugh and Maibach 2005; Prausnitz et al. 2004; Williams 2003).

Different approaches to physically circumvent the stratum corneum barrier or to enhance penetration of active substances into and/or across the skin using chemical penetration enhancers (Cleary 2013; Kumar 2011; Mathur et al. 2010) are already employed by pharmaceutical and cosmetic industries. Regrettably, they are often associated with irritation or toxicity issues. Therefore, safe carriers that enable or enhance percutaneous penetration are of great interest (Bolzinger et al. 2012; Cevc 2004). The growing relevance of microemulsion systems as carriers for cutaneous drug delivery, superior to conventional topical formulations, is well documented (Kogan and Garti 2006; Sintov and Shapiro 2004). Microemulsions are isotropic, transparent,

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thermodynamically stable colloidal systems consisting of water phase, oil phase, and sufficient concentrations of an appropriate surfactant in combination with a suitable cosurfactant. The microstructure of such systems is organized on the level below 100 nm providing a large interface which is available for solubilization of drug molecules (Djekic et al. 2012; Djekic et al. 2011; Fanun 2009; Heuschkel et al. 2008). Current investigations are focused on development of biocompatible microemulsion systems comprising GRAS excipients (Djekic and Primorac 2008; Fanun 2011). Microemulsions have low viscosity that compromises their application on the skin (i.e., they spread beyond the intended area). Promising gel-like systems derived from microemulsions, named as *microemulsion-based gels* (MBGs) (Atkinson et al. 1988), are suitable to overcome this disadvantage. Although they are two-phase systems comprising an oil phase and a water phase, they are also referred as *microemulsion-based organogels*, since a nonpolar phase (often an organic solvent) is the main component (Luisi et al. 1990; Rees et al. 1998). Liquid phase of MBGs, similar to organogels, is immobilized by a three-dimensional network composed of self-assembled molecules of a gelling agent. MBGs originate from water-in-oil microemulsions or reverse micelles, which may be gellated by using a suitable amphiphile (e.g., lecithin, nonionic surfactants) and/or a polymer (e.g., gelatin, poloxamers) (Sahoo et al. 2011; Vintiloiu and Leroux 2008). Macroscopically, MBGs are transparent or opaque systems, depending on their composition and microstructure. They are thermodynamically stable systems since the gelling agent undergo self-assembly, which results in the decrease in the total free energy. The physicochemical investigation of these systems, applying a variety of techniques such as nuclear magnetic resonance (NMR) spectroscopy, Fourier transform infrared (FTIR) spectroscopy, differential scanning calorimetry (DSC), dynamic shear viscosity measurements, and photon correlation spectroscopy (PCS), revealed viscoelastic and thermoreversible character (Shapiro 2011; Tanaka 2006). The viscoelastic MBGs behave like solids at lower shear rates and hence show an elastic property. As the shear stress is increased, the physical interacting points among the fiber structures of a gelling agent start getting weakened until the shear stress is high enough to disrupt the interactions among the fiber structures, when MBGs start flowing. On heating above a critical temperature, the supramolecular architecture is melted and individual molecules (or separated strands of aggregated molecules) are re-dispersed in the bulk solution and MBGs start flowing. As the system is subsequently cooled down, physical interactions among the gelator molecules prevail and the viscoelastic organogel reverts back. The nature of MBG networks varies in accordance to the gelling agent(s) and intermolecular interactions. More detailed information about molecular organization, specific interactions, and internal mobility of constituents, obtained by applying sophisticated techniques, such as multiple-quantum (MQ) spectroscopy, pulse field gradient (PFG) technique, and magnetic resonance imaging (MRI), enable deeper understanding and development of microstructure models for MBGs (Shapiro 2011).

Scartazzini and Luisi with coworkers (Luisi et al. 1990; Scartazzini and Luisi 1988), in the late 1980s, were first who described a formation of a jellylike state from initial nonviscous solution of lecithin in organic solvent on an addition of trace amounts of water. By now such lecithin-based MBGs have been studied by many authors worldwide, particularly those with the relevance as drug delivery vehicles (Belgamwar et al. 2008; Murdan 2005b; Rees et al. 1998; Shchipunov 2001; Willimann et al. 1992). Ready-to-use lecithin-based MBGs (DiffusiMax®, Transderma®, Phlojel®) are already available on the world market and used for compounding of topical formulations with a wide variety of drugs (e.g., nonsteroidal anti-inflammatory drugs (NSAIL), local anesthetics, hormones). Gelatin-stabilized MBGs were first reported in the late 1980s by the groups of Haering and Luisi (1986) and Quellet and Eicke (1986). They were originally obtained when gelatin was added into water-in-oil microemulsions comprising anionic surfactant sodium bis(2-ethylhexyl)

sulfosuccinate (AOT), isooctane, and water (Haering and Luisi 1986; Luisi et al. 1990; Kantaria et al. 1999).

Gelatin-stabilized MBGs have been investigated for iontophoretic transdermal drug delivery (Kantaria et al. 1999, 2003). Advantages of MBG vehicles are ease of preparation as well as the possibility to accommodate both hydrophilic and lipophilic compounds. MBGs tolerate relatively high concentrations of additives without undergoing the phase transitions, often seen in water-in-oil microemulsions. The important benefit of MBGs is a high potential for enhancement of percutaneous penetration of drugs which is mainly ascribed to well-known penetration-enhancing effect of typical MBG components, i.e., surfactants, phospholipids, and fatty acid esters (Rowe et al. 2009). However, permeation-enhancing properties may also depend on the formulation characteristics such as rheological behavior and structure of the vehicle, the physicochemical properties of the drug molecule (e.g., size, chemical structure, solubility in oil and water), thermodynamic activity of the drug within the vehicle, concentration gradient toward the stratum corneum, and histological characteristics of the skin (Barry 2002; Cevc 2004).

17.2 Lecithin Organogels (LOs)

17.2.1 Composition and Formation of LOs

LOs are three-component systems comprising lecithin (gelling agent), oil phase, and water phase. Lecithin (1,2-diacyl-*sn*-glycero-3-phosphocholine) belongs to a class of phospholipids. It is a naturally occurring mixture of diglycerides of stearic, palmitic, and oleic acids linked to phosphatidylcholine (Gunstone et al. 1994; Wendel 1995). The main natural sources of lecithin are soybean and egg yolk[1] (Szuhaj 1989). It is brown to light yellow semisolid to powder practically insoluble in water and polar solvents, as well as in cold vegetable and animal oils (Sheng 2009). Lecithin is the important constituent of biological membranes (Hanahan 1997). It is a GRAS excipient widely used as an emulsifier, stabilizer, solubilizer, and penetration enhancer in pharmaceutical and cosmetic products (Wade and Weller 1994). Lecithin is able to self-assemble into three-dimensional network structure which leads to gelation of organic fluids in a presence of a low amount of water (Shchipunov 2001; Shchipunov et al. 2001). The concentration of lecithin in LOs ranges from 50 to 200 mM.

Only highly purified lecithin of natural origin enables the formation of LOs (Scartazzini and Luisi 1988). In this case a very small amount of water is needed and no guest polymer is necessary for inducing the gelation. However, the process of organogel formation fails if the purity of naturally occurring lecithin is below 95 % (Liu 2005; Kumar and Katare 2005; Schurtenberger et al. 1990). Also, hydrogenated lecithin and synthetic lecithin are not suitable to form LOs. Lecithin-based organogels have been formed with many organic solvents (e.g., linear, branched and cyclic hydrocarbons, ethers and esters, fatty acids, and amines) (Sahoo et al. 2011; Vintiloiu and Leroux 2008; Yurtov and Murashova 2003); however, limited members are biocompatible. Of particular interest are fatty acid esters (e.g., isopropyl myristate (IPM), isopropyl palmitate (IPP), isostearyl isostearate (ISIS), ethyl oleate (EO)) as emollients with very good spreadability (Belgamwar et al. 2008; Dreher et al. 1997; Moore 1982; Murdan 2005b; Rees et al. 1998; Schurtenberger et al. 1990; Willimann et al. 1992). The third component of LOs is water phase. The content of water varies depending on the oil phase.

Typically, the molar ratio of water to lecithin (w_o) is from 1 to 12 (Scartazzini and Luisi 1988). The ratio of number of water molecules per lecithin molecules governs the phase behavior of the lecithin/oil/water systems (Shchipunov and Shumilina 1995, 1996; Schurtenberger et al. 1990; Schurtenberger and Cavaco 1994). The

[1] Commercially available under trademarks Epicuron®, Topcithin® (Degussa Texturant Systems Deutschland GmbH & Co./Lucas Meyer GmbH & Co., Germany), and Lipoid® (Lipoid GmbH, Germany).

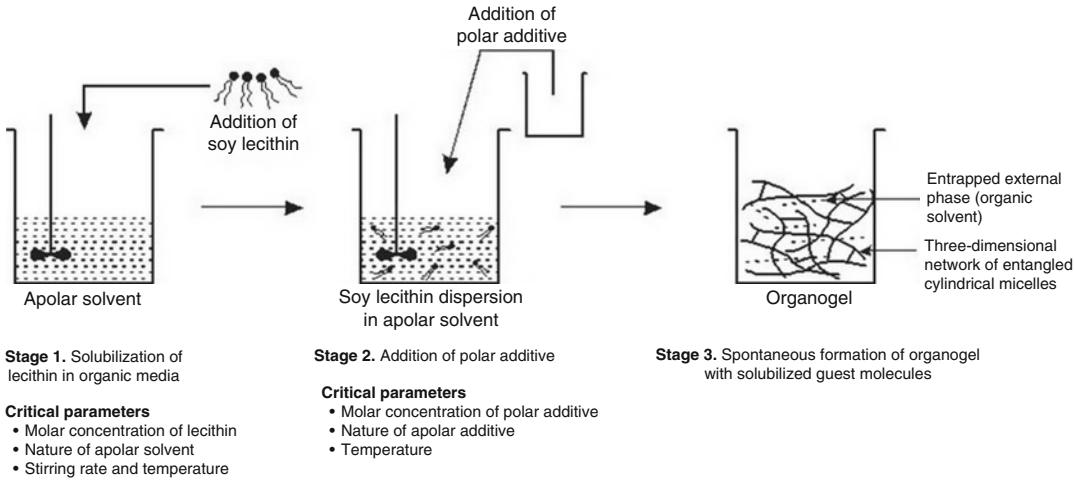

Fig. 17.1 Schematic diagram of the preparation of lecithin organogels (Kumar and Katare 2005)

organogels form in a narrow range of concentrations of the constituents (Raut et al. 2012). The percentages of the components producing the organogel depend strongly on the dryness of lecithin. It is a hygroscopic substance; therefore, it always contains a portion of water which lowers the content of additional water required for organogel formation (Shchipunov and Shumilina 1995; Trotta et al. 1999). Apart from water, cosolvents such as glycerol, ethylene glycol, propylene glycol, and formamide also may form LOs, and they are often components of the aqueous phase. Interestingly, ethyl alcohol and diethylene glycol do not promote LO formation (Shchipunov and Shumilina 1995, 1996). LOs are susceptible to oxidation; therefore, antioxidants are usually incorporated (Raut et al. 2012).

Preparation of LOs is a simple two-stage procedure (Fig. 17.1).

Lecithin dissolves in the oil phase. Solution of lecithin transforms into highly viscous gel by adding water phase under mild stirring. The preparation method is suitable for thermolabile drugs (e.g., peptides and proteins) (Kumar and Katare 2005; Willimann and Luisi 1991). Ready-to-use LOs (e.g., Phlojel® Ultra, J.A.R. Pharmaceuticals, Canada[2]) are worldwide marketed bases for compounding of topical formulations. Apart from that, *premium lecithin organogels* (Transderma® PLO[3] Ultramax Gel, Transderma Pharmaceuticals, Canada[4]) are being marketed as ready-to-use intradermal bases with recommendations for application in achieving improved percutaneous bioavailability. Premium lecithin organogels are specially developed to target local tissues and achieve local therapeutic drug levels while minimizing blood-level-related toxicities (http://www.transderma.com/transderma-plo-gel.html).

17.2.1.1 Mechanism of Gelation

Lecithin in oil phase self-assembles into reversed spherical micellar aggregates. Upon addition of small amount of water, or other suitable polar solvent, reverse micelles elongate into giant cylindrical micelles (in the literature often called *reverse wormlike*, *spaghettilike*, or *polymer-like* micelles). It was suggested that the formation of such lecithin reverse micelles is the result of hydrogen bonding between the phosphate group of the lecithin molecule and a polar solvent that creates linear network. Elongation of micelles and their subsequent overlapping, interpenetration, entanglement, and branching result in formation of three-dimensional "polymer-like"

[2] http://www.jarpharma.com/.

[3] PLO is abbreviation for premium lecithin organogels.

[4] http://www.transderma.com/.

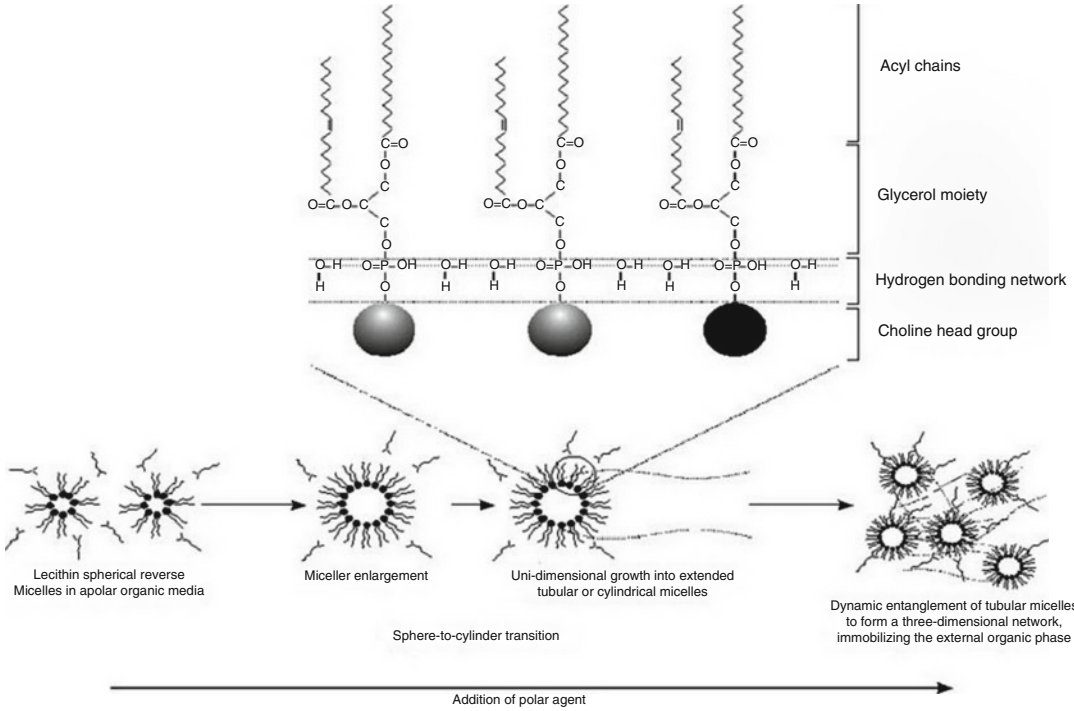

Fig. 17.2 Formation of a three-dimensional network of reverse cylindrical micelles in lecithin organogel, involving hydrogen bonding between lecithin and polar solvent molecules (Kumar and Katare 2005)

network which immobilizes the bulk oil phase (Fig. 17.2) (Capitani et al. 1996; Kumar and Katare 2005; Luisi et al. 1990; Raut et al. 2012; Scartazzini and Luisi 1988; Schurtenberger et al. 1990; Schurtenberger and Cavaco 1994; Shchipunov 1997).

The network is transient, i.e., characterized by the continuous breaking and recombination of the aggregates that is typical for weak gels. Recent reports implicate that the homogeneous LOs represent rather complicated systems comprising at least three subsystems: (1) linear polymer-like micelles, (2) branched micellar aggregates, and (3) a mixture of entities possessing various relaxation times. Nevertheless, the sequential change of micellar shape was followed by abrupt viscosity enhancement (10^4–10^6 times) (Shapiro 2011; Shchipunov 2001; Shchipunov et al. 2001; Shumilina et al. 2000). Glycerol was found to provide maximal viscosity of the ternary system at the lowest concentration ($w_o = 1.7$–1.9), followed by water ($w_o = 3.6$–3.8), formamide ($w_o = 3.6$–4.8), and ethylene glycol ($w_o = 5$) (Shchipunov and Shumilina 1996). Any additive (e.g., surfactant, cosolvent, drug) has an effect on the lecithin organogels structure and physicochemical properties. Macroscopically, LOs are transparent or opaque jellylike systems. They are isotropic, thermodynamically stable, and thermoreversible systems (sol-to-gel transition temperature is around 40 °C) (Kumar and Katare 2005; Schurtenberger et al. 1990; Shchipunov 1995; Zhao et al. 2006). Drug incorporation usually reduces LO viscosity. LOs also demonstrate high sensitivity to external influences such as pressure or electric field, which may represent a suitable way for controlling their structure. As the pressure was decreased, the viscosity and conductivity increased dramatically suggesting an enhanced attractive interaction between reverse polymer-like micelles or their enlargement (Shapiro 2011). The external electric field also induced a notable reversible increase in the viscosity of LOs (Shchipunov and Schmiedel 1996); however, such electrorheological effects are poorly investigated.

17.2.2 Percutaneous Permeation Enhancement Potential of LOs

LOs may be efficient vehicles for transdermal transport of various drugs. Many studies have explored cutaneous delivery potential of LOs for variety of active substances (e.g., NSAIDs, anticholinergics, β-adrenergic agonists, anticancer drugs, antihypertensives, psychopharmaceuticals). LOs are found desirable drug delivery vehicles and are capable of providing an appropriate drug release pattern. In a very recent study (Esposito et al. 2013), LOs were studied as vehicles for solubilization and percutaneous delivery of a retinoic acid derivative fenretinide (4-hydroxypropyl phenyl retinamide). The influence of different types of oil, content of water, and presence of hyaluronic acid was studied on gel properties. LOs were prepared by dissolving lecithin (200 mM) in IPM, IPP, or ISIS and adding water, under magnetic stirring. The drug was easily solubilized in the organogels avoiding thermal stress.

The *in vitro* drug release from the prepared LOs was evaluated by Franz cell coupled with a synthetic nylon membrane. It was found that diffusion coefficients of fenretinide incorporated in LOs are at least 20-fold higher with respect to conventional topical formulations. The investigated fenretinide-loaded LOs were promising for percutaneous treatment of skin carcinomas. A comparative percutaneous permeation study of corticosteroid clobetasol propionate from a conventional gel, the investigated LOs comprising soybean lecithin, IPM, and mixture of distilled water and glycerol, and a plain drug solution, was carried out through excised rat skin using Franz diffusion cell (Surjyanarayan et al. 2010). Furthermore, the effect of formulation variables on the percutaneous permeation profile of the drug from the LO formulations was determined through excised rat skin. A significant decrease in drug release from formulations was observed with increase in concentration of lecithin from 32 to 62 % w/w. Viscosity and transparency of formulations were observed to be directly affected by lecithin concentration. The drug release increased remarkably as the loading was increased from 0.05 to 0.1 % w/w. Increased oil concentration leads to increase in drug release from the formulation. The optimized formula of the LOs contained 0.05 % w/w clobetasol propionate, 32 % w/w lecithin, 66 % v/w IPM, and 2 % v/w water/glycerol (1:1). Skin retention of the drug through comparative cutaneous deposition analysis was found to be 7.4 % more for the developed LOs than marketed gel. This system was capable of providing sustained drug release up to 10 h. Thus optimized LO vehicle facilitates the penetration of clobetasol propionate through the skin at sustained manner to obtain optimum drug concentration on the site of action (within the skin) and avoid systemic side effects.

Percutaneous penetration of the different drugs by using LOs vehicles has been evaluated in several *in vitro* and *in vivo* studies. Dreher and coworkers (1997) have investigated *in vitro* percutaneous penetration of indomethacin and diclofenac, dissolved in the soybean lecithin/IPP/water microemulsion gel. The permeation study through a human full-thickness skin was performed by using a Franz-type diffusion cell. Indomethacin and diclofenac were dissolved in a 250 mM LOs ($w_o = 0$) at half-saturation (35 mg/ml). Neat IPP, in which the drugs were administered at saturation (1.5 mg/ml for indomethacin, 0.1 mg/ml for diclofenac), served as comparison. The permeation rates of indomethacin or diclofenac dissolved in the LOs were similar (about 1 $\mu g h^{-1} cm^{-2}$). In addition, the permeation rates of the drugs dissolved in IPP alone were also similar (about 0.2 $\mu g h^{-1} cm^{-2}$), but smaller, compared to the LOs. By calculating the corresponding permeability coefficients for indomethacin and diclofenac, 4 and 100 times higher permeability coefficients, respectively, have been obtained from IPP compared to the LO. The obtained results were related with the drastically different solubilities of the two drugs in the different formulations as well as with an unfavorable LO/*stratum corneum* partition coefficient of indomethacin and diclofenac compared to the corresponding partition coefficient for IPP alone. This effect seems to be more pronounced for diclofenac than for indomethacin. In a related study reported by Fujii et al. (1996), LOs were

developed using lecithin and fatty acid esters. The authors selected 20 fatty acid esters.

Five fatty acid ester groups, octanoate, isononanoate, myristate, palmitate, and stearate, were selected. The total number of carbon atoms in the fatty acid esters ranged from 17 to 34. The solubility of indomethacin was higher in fatty acid esters containing fewer carbon atoms. The permeation rate of the model drug from a fatty acid ester suspension through excised hairless rat skin (~1–3 μg/cm^2/h) was proportional to its solubility in the suspension. LOs were then formulated from esters by the addition of a phospholipid. The permeation rates of indomethacin through the excised hairless rat skin from LOs were from 4 to 20 μg/cm^2/h which were higher than those from the suspension. The permeation experiments conducted indicated that the permeation of the indomethacin was higher from the LOs which had side chains on both fatty acid and alcohol moieties. Although the mechanism was not clarified, it was speculated that the structures of these compounds enabled them to interact with the components of the *stratum corneum* and, consequently, to enhance permeation of drugs through the skin. LOs comprising lecithin, EO, and water were found to be more effective for delivery of aceclofenac when compared with hydrogels (Shaikh et al. 2006). The permeation of scopolamine and broxaterol from lecithin/IPP/water LOs was investigated *in vitro* using human skin obtained from plastic surgery and Franz diffusion cell. The transport rate of scopolamine from the lecithin gel into the receptor phase was about ten times higher than the transport rate from an aqueous scopolamine solution (Figs. 17.3 and 17.4).

Interestingly, the transport rates of the drug (scopolamine or broxaterol) from lecithin/IPP gel and from a lecithin solution (the system prior to the addition of water) were similar. This shows that gelation did not influence the rate of drug transport through the skin. Enhanced drug permeation through the skin was assigned to the effects of lecithin on the skin. Lecithin (from the organogel or the solution prior to gelation) was thought to penetrated into the skin, interacted, and disorganized the lipid layers of the *stratum corneum*, thus increased a drug movement into and through the skin (Fig. 17.5) (Willimann et al. 1992).

Lecithin/IPM/water organogels have also been tested as transdermal delivery vehicles for piroxicam to reduce the drug's adverse effects and to avoid its first-pass metabolism when administered orally (Pénzes et al. 2005). *In vitro* investigations in diffusion cells showed that 40 % of the drug present in the vehicle had permeated through excised abdominal rat skin after 8 h.

Aboofazeli et al. (2002) have evaluated mechanistic effects of formulation components on *in vitro* transdermal permeation of a calcium channel blocker nicardipine hydrochloride (NC-HCl) through the excised skin of a hairless guinea pig and human *stratum corneum* using Franz diffusion cell. Vehicles investigated included pure solvents alone and their selected blends. Propylene glycol (PG) was selected as the main vehicle in the development of a transdermal product. The skin permeation was then evaluated by measuring the steady-state permeation rate (flux), lag time, and the permeability constant of NC-HCl. The results showed that no individual solvent was capable of promoting NC-HCl penetration. Permeation profiles of the drug through hairless guinea pig skin using saturated solutions of drug were constructed. Among the systems studied, the ternary mixture of PG/oleic acid (OA)/dimethyl isosorbide (DMI) and binary mixture of PG/OA showed excellent flux. The flux value of the ternary system was nearly three times higher than the corresponding values obtained for the binary solvent. A similar trend also was observed for the permeation constant, while the values of lag time were reversed. The ternary mixture was then selected as a potential absorption enhancement vehicle for the transdermal delivery of drug. In general, higher fluxes were observed through hairless guinea pig skin as compared with the human *stratum corneum*. Based on the results obtained from the release study of NC-HCl from saturated solutions of the drug, a LO composed of soybean lecithin, PG, OA, DMI, and IPM was developed as a potential vehicle for transdermal delivery of NC-HCl. *In vitro* percutaneous penetration studies from this LO revealed that it has drug permeation-enhancing potential and could

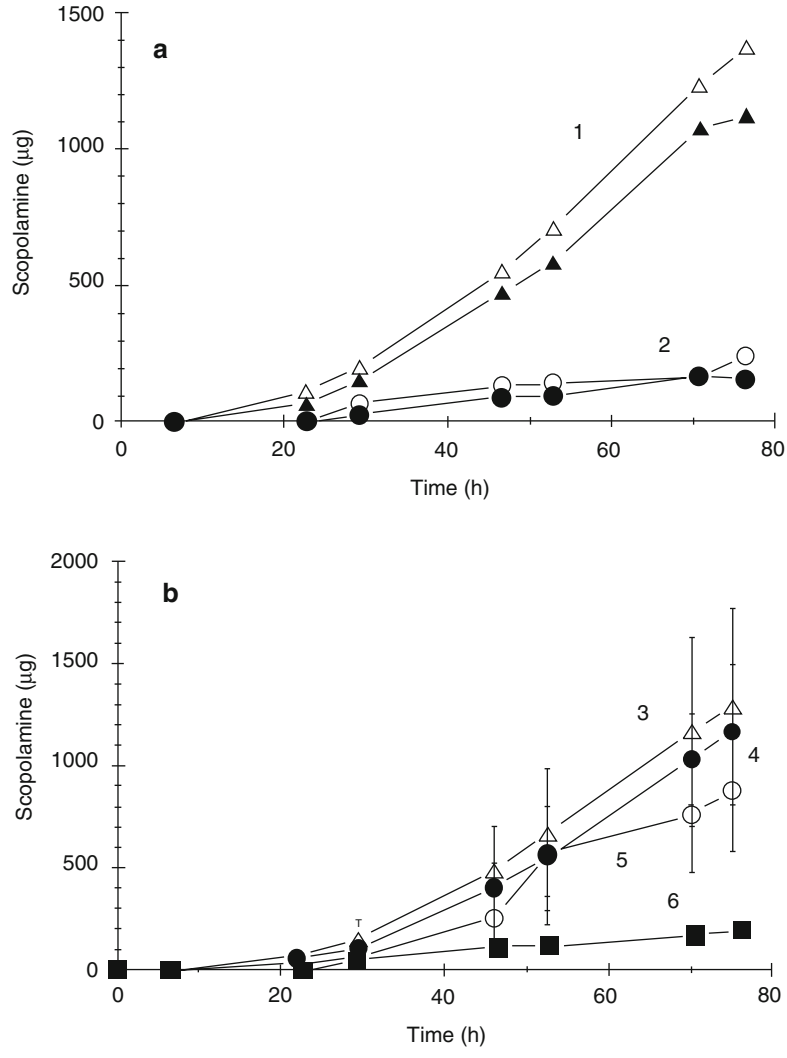

Fig. 17.3 Transport of scopolamine through human skin as a function of the matrix system used. (**a**) Soybean lecithin (200 mM)/IPP (w_o 3) (*curve 1* Δ, ▲) or 50 mM HEPES buffer pH 6.8 (*curve 2* ○, ●). *Curves 1* and *2* represent data from two individual experiments. (**b**) Soybean (200 mM) lecithin/IPP: w_o 4 (*curve 3* Δ), w_o 3 (*curve 4* ●), and w_o 0 (*curve 5* ○). Each data point represents the mean values and standard deviations of nine measurements with six skin samples. *Curve 6* (■) is from data with a commercial scopolamine plaster (Scopoderm TTS 1.5 Ciba-Geigy). Aliquots of 80 µl (for gel) or 400 (for buffer solution) with an initial scopolamine concentration of 39 mg/ml (corresponding to a total amount of scopolamine of 3.1 or 15.6 mg, respectively) were placed in the donor compartment. The Scopoderm TTS contained a total of 1.5 mg scopolamine (Willimann et al. 1992)

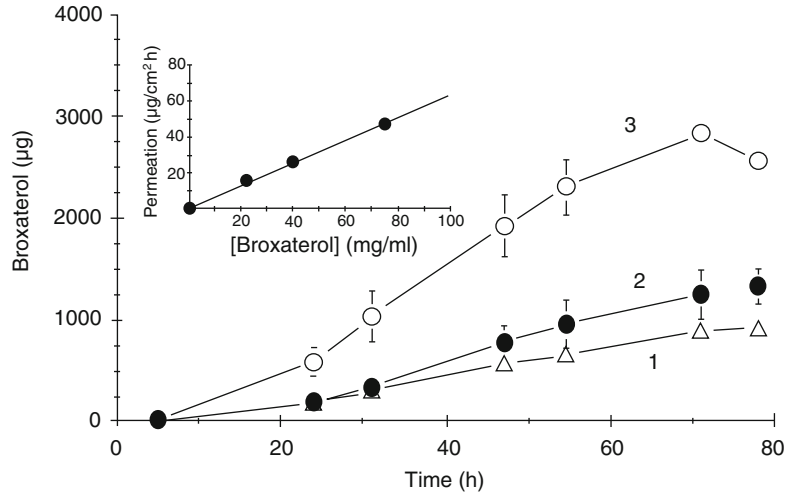

Fig. 17.4 Transport of the drug through human skin in vitro from 200 mM soybean lecithin/IPP (w_o 0) initially containing broxaterol at 23 (*curve 1*; Δ), 40 (*curve 2*; ●), or 75 (*curve 3*; ○) mg/ml. The dependence of the transport rate on the initial broxaterol concentration is shown in the inset (Willimann et al. 1992)

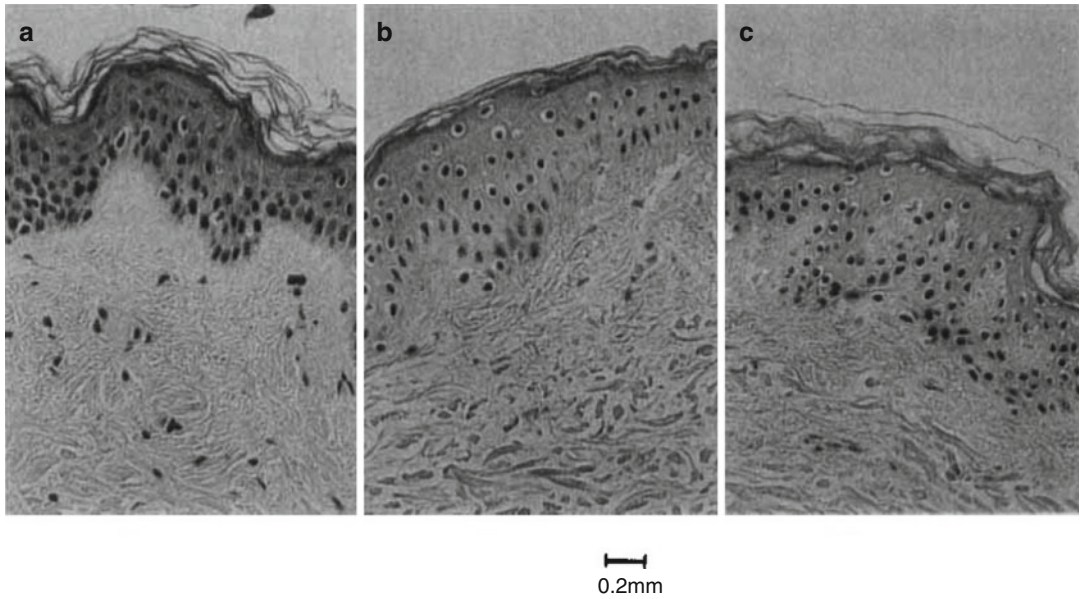

Fig. 17.5 Light micrographs of human chest skin: (**a**) untreated, immediately after thawing the frozen skin; (**b**) after treatment with 200 mM soybean lecithin/IPP LO ($w_o = 3$) for 3 days; (**c**) after treatment with 0.4 % aqueous NaCl solution for 3 days (control) (Willimann et al. 1992)

be a promising matrix for the transdermal delivery of NC-HCl. Furthermore, higher permeation rates were observed when nicardipine free base was incorporated into the LO matrix instead of hydrochloride salt.

Nastruzzi and Gambari (1994) observed a suppression of subcutaneous tumor growth in nude mice xenografted with the highly tumorigenic cell line FH06T1-1 by treatment with a LO containing a substance with an antitumor activity tetra-benzamidine. When the LO was applied away from the affected region, the tumor continued to grow, demonstrating lower systemic versus local effects of the system. The in vivo permeation of piroxicam was assessed by measuring the drug's anti-inflammatory effect versus the inhibition of carrageenan-induced rat paw edema (Agrawal et al. 2004). Drug-loaded LO was applied to the shaved abdominal skin of albino rats, and carrageenan suspension was injected into the right hind paw of the rats after 1 h. The paw volume was measured for treated and control rats. The latter received applications of lecithin organogels without drug. Application of the piroxicam-loaded LOs was found to significantly inhibit edema and changes in the hind paw volume compared with the control formulation, thus indicating that piroxicam had permeated through the skin following topical application, had been absorbed into the systemic circulation, and had acted away from the application site. The investigated LOs were superior to the marketed product in inhibition of rat paw edema. Several clinical trials on patients suffering of various musculoskeletal diseases (osteoarthritis, lateral epicondylitis, sprains, etc.) and treated transdermally with 1–2 % diclofenac in LO revealed significant improvements in terms of analgesic efficacy compared to placebo (Grace et al. 1999; Mahler et al. 2003; Spacca et al. 2005). Methyl nicotinate (0.5 % w/w) has been formulated in a soybean lecithin/IPM/water organogel and tested in vivo in human subjects (Bonina et al. 1995). Almost complete percutaneous absorption in a short period of time was coupled by the induction of erythema, which may be attributable to a disruption of the *stratum corneum*. Percutaneous permeation enhancement by the constituents of LOs, lecithin, and fatty acid esters (IPP, IPM, EO) has been widely investigated (Cevc 2004;

Mahjour et al. 1990; Sato et al. 1988; Stroppolo et al. 1991). These substances can penetrate into the skin creating highly permeable pathways for permeation of the drug into the skin layers. Permeation enhancement effect could be ascribed to fluidization of the lipid bilayers of the *stratum corneum*, with eventual extraction of lipid components and disruption of corneocytes, as well as increased hydration of the epidermis.

Dreher et al. (1997) investigated the interactions between lecithin/IPP MBGs and the human *stratum corneum* in order to evaluate the role of lecithin and the oil phase as permeation enhancers, by using FTIR and DSC. FTIR spectra of isolated human *stratum corneum* exposed to lecithin/IPP gel or to neat IPP for 5 days showed absorption peaks related to IPP but not to lecithin. IPP had permeated into the *stratum corneum*, whereas lecithin had not. In addition, permeation of IPP into the *stratum corneum*, from the gel and from neat IPP, occurred to the same extent. This shows that lecithin did not enhance IPP permeation into the stratum corneum. DSC analysis on the *stratum corneum* exposed to the investigated LO or to neat IPP indicates disruptions of the lipid bilayers in the *stratum corneum* which were primary ascribed to the oil phase. However, more systematic studies should be carried out to clarify whether and to what extent the oil phase, concentration of lecithin, amount of water, as well as chemical structure, hydrophobicity, molecular weight, and charge density of the drug molecule affect the transport rate. Apart from this, LOs also provide occlusion and smooth feel on the skin (Kumar and Katare 2005; Vintiloiu and Leroux 2008). Furthermore, the results of skin compatibility studies, *in vitro* as well as *in vivo* in animals and in man, showed no significant acute and cumulative irritation potential of LOs (Dreher et al. 1997; Kumar and Katare 2005; Shchipunov 2001). Agrawal et al. (2004) applied piroxicam-loaded lecithin/IPM LOs to the shaved backs of rabbits and found that the gels did not produce any sensitization during a 3-day observation period. Dreher and coworkers (Dreher et al. 1995, 1996) investigated the acute (48-h patch test) and cumulative (21-day repeated insult patch test) irritation potential of LOs in human volunteers and showed that the gels caused little irritation. In this study, 2 of the 151 volunteers taking part in the acute test suffered from slight erythema at the application site. In the cumulative irritation test, significant irritation was observed in 8 of the 16 volunteers following 13 days of continuous gel exposure to the same skin site. In practice, the site of gel application would be rotated on consecutive days; the cumulative insult test was conducted to assess that the irritation in case the gel was misused and applied to the same skin site repeatedly. These investigations increase the likelihood that lecithin gels containing a biocompatible liquid will be used as transdermal or topical drug delivery vehicles.

17.3 Pluronic®/Lecithin Organogels (PLOs)

The major limitation in formation of LOs is the requirement of highly purity grade lecithin which is expensive and it is difficult to obtain in large quantities. Inclusion of polyoxyethylenepolyoxypropylene-polyoxyethylene block copolymers (poloxamers (EP, USP)) (commercially known as Pluronic®, Lutrol®, or Kolliphor® P Grades copolymers (BASF, Germany)) as cosurfactants makes the organogelling feasible with lecithin of relatively lesser purity. Biocompatible PLOs were being designed by compounding pharmacists Jones and Kloesel in the USA in the early 1990s as vehicles for extemporaneous preparation of topical formulations (Loyd 2003). Their transdermal drug delivery potential is currently of great interest to the pharmaceutical scientist (Almeida et al. 2012; Belgamwar et al. 2008; Kumar and Katare 2005; Murdan 2005a).

17.3.1 Composition and Formation of PLOs

PLOs are viscoelastic gels derived from LOs by partial replacement of the natural lecithin by a poloxamer (e.g., Pluronic® F127). Poloxamers are long-chain ABA block copolymers widely used as emulsifying agents, solubilizing agents,

and wetting agents in drug delivery systems for subcutaneous, intranasal, vaginal, rectal, ocular, parenteral, dermal, and transdermal administrations, in concentrations from 15 to 50 % (Collett 2009). They are nontoxic, biocompatible excipients with weak immunogenic properties. In relatively highly concentrations, poloxamers exhibit a sol–gel transition phase below or near the physiological body temperature and a gel–sol transition at ~50 °C (Dumortier et al. 2006). The copolymer is dissolved in aqueous phase of PLOs usually in concentration of 20 %. The oil phase of PLOs, usually IPP or IPM, is used for lecithin solubilization. The apolar phase in PLOs usually constitutes ~20 % v/v. The percentage of aqueous phase in PLOs is up to 80 % v/v; therefore, the introduction of a preservative (e.g., sorbic acid/potassium sorbate) is recommended (~0.2–0.3 % w/w)) (Belgamwar et al. 2008; Murdan 2005a).

Common method of preparation of PLOs is by admixing an aqueous phase containing poloxamer and an oil phase containing lecithin, until the mixture converts into the homogeneous gel (Choukse and Sangameswaran 2012; Murdan 2005a). The polymer is dissolved in ice cold water and the solution is refrigerated overnight (at low temperatures the solution is in liquid state, while at higher temperatures it gels). Lecithin is also dissolved previously in the oil phase. In recent years, there has been an increase in the use of marketed PLO kits (e.g., Transderma® Hydrophylic (30 % pluronic/water) + Transderma® Organic (lecithin/IPP), Xenex Labs, Canada[5]; PHLOJEL® Hydrophilic + PHLOJEL® Organic, J.A.R. Pharmaceuticals, Canada[6]; PLO Gel Kit, Pharmedica Enterprise, Malaysia[7]) containing well-balanced aqueous phase (e.g., Pluronic® F127, potassium sorbate, water) and oil phase (usually comprising lecithin, IPP, and sorbic acid). Ready-to-made PLO bases are also available on the market (e.g., Transderma® PLO Ultramax Gel, Transderma Pharmaceuticals, Canada; DiffusiMax®, Maxima Pharmaceuticals, Canada[8]; Phlojel® PM, J.A.R. Pharmaceuticals, Canada) for compounding of topical preparations with variety of drugs. Incorporation of the drug substances depends on their solubility. The lipophilic drugs dissolve in the oil phase, while the hydrophilic drugs dissolve in the water phase, before mixing the two phases (PLO GEL. Application in Pharmacy Compounding, Compilation 2011). Dissolution of drug in aqueous phase may require cosolvents such as propylene glycol or ethoxydiglycol (Loyd 2010). The premixed PLO vehicles can be used to levigate the drug followed by dilution with remaining amount of PLO vehicle. In the case of a highly crystalline substance (e.g., ketoprofen), a small amount of levigating agent, such as alcohol or propylene glycol, is usually added to the powder and levigated. Ready-to-made PLOs are usually recommended in cases of drug combinations and for drugs that pose a problem of gelation using the conventional preparation approach (PLO GEL. Application in Pharmacy Compounding, Compilation 2011).

PLOs are yellow colored and opaque viscoelastic thermodynamically stable systems. Similar to LOs, PLOs consist of entangled tubular reverse micelles which form temporal three-dimensional structures. The copolymer forms multimolecular aggregates and immobilizes the water phase (Desai et al. 2007). PLOs exhibit temperature-dependent viscosity. PLOs convert into liquid by refrigerating, which may separate into oil and aqueous phases after sufficient time. Also, as the preparation is rubbed into the skin and warms up, it may become slightly more viscous and resistant to rubbing (Loyd 2003). Although the application of PLOs as drug delivery vehicles in medical practice follows their discoveries, their physicochemical properties as well as detailed information on morphology, molecular organization, specific interactions, and internal mobility of the constituents are scarcely investigated.

[5] http://www.xenexlabs.com/.

[6] http://jarpharmaceutical.com/.

[7] http://www.plo-gel.com/Main/index.php.

[8] http://www.maximapharmaceuticals.com/.

17.3.2 Percutaneous Permeation Enhancement Potential of PLOs

The coexistence of organic and aqueous phases as well as a large interfacial area, which are available for drug solubilization, along with long-term stability, makes PLOs useful vehicles for topical and transdermal applications of both hydrophobic and hydrophilic molecules, including substances with a higher molecular weight and higher water solubility. The related references are available at the International Journal of Pharmaceutical Compounding website.[9]

The organized microstructural matrix of the PLOs, their well-balanced hydrophilic and lipophilic characteristics and high solubilizing capacity, and possible interaction of the lipid excipients with skin tissues implicate that such vehicles may efficiently partition with the skin and enhance the transport of drug molecules into and across the skin. In addition, PLOs have been shown, *in vitro* and *in vivo*, to modulate the release and percutaneous permeation of the incorporated drug (Almeida et al. 2012; Belgamwar et al. 2008; Kumar and Katare 2005; Murdan 2005a). *In vitro* drug release studies show that the increase in concentration of lecithin decreases the cumulative percentage of flurbiprofen released, which might be due to the extensive formation of a network-like structure with very high viscosity (Belgamwar 2009). In another study, however, *in vitro* release study of a topical PLO formulation of methimazole revealed that the components of the vehicle do not pose an obstacle to the drug release (Morales et al. 2009). According to Grace et al. (1999) administration of ketoprofen in a PLO gel offered convenience, produced fewer side effects, and alleviated pain in a specific location. However, Dowling and coworkers (Dowling et al. 2004) reported low and highly variable relative bioavailability of ketoprofen when the drug was administered as a single dose in a PLO-based ketoprofen 20 % gel. In the third study, PLO was adapted to contain fish oil, ketoprofen, or both, with 1,8-cineole as penetration enhancer, and used to determine the *in vitro* permeation from infinite and finite dosing protocols across full-thickness porcine skin. A PLO was capable of delivering *via* a repeat finite dosing regimen, although there is evidence for the sustained release of ketoprofen (Richards et al. 2006).

Efficiency of PLO as a topical and transdermal delivery system has been evaluated by carrying out various types of studies conducted by clinicians. PLO containing 2 % of diclofenac was applied three times daily for 2 weeks (Burnham et al. 1998) and for 1 week (Grace et al. 1999) to the affected site for the treatment of osteoarthritis of the knee and of lateral epicondylitis, respectively. Following the application of a diclofenac-loaded PLO, pain was significantly lower than during the periods of pretreatment, washout, or placebo PLO administration (Burnham et al. 1998; Grace et al. 1999) and stiffness was reduced (Grace et al. 1999). However, diclofenac levels in the blood were not measured and drug absorption into the systemic circulation cannot be assumed. In a related study, the PLO formulation (Pluronic® F127, lecithin, IPM, water, sorbic acid, and potassium sorbate) containing flurbiprofen showed a statistically significant anti-inflammatory activity, and it is a nonirritant to the skin (Choukse and Sangameswaran 2012). The efficacy of a PLO as a transdermal delivery vehicle for ondansetron, a 5-HT receptor antagonist, was evaluated in 12 healthy volunteers against pain, mechanical hyperalgesia, and flare following (Giordano et al. 1998). The PLO gel was reported to be a good topical vehicle for ondansetron, producing significantly greater pain reduction. However, no control formulations were used, so the benefit of the PLO over a simple ondansetron solution cannot be ascertained. The formulation of testosterone has been successfully prepared by incorporating the therapeutically effective amounts of micronized testosterone in a PLO gel. Plasma concentrations of testosterone increased after 20–30 days and reached an apparent steady state during the administration period (Kryger 2002). Application of ketamine hydrochloride (10 %) with a PLO vehicle directly to the site of pain lowered the sympathetic or neuropathic pain and enables the avoidance of the

[9] http://www.ijpc.com/.

undesirable side effects (Finch et al. 2009; Flores and Crowley 1998). Glisson et al. (2005) performed an *in vivo* study in humans and concluded that the absolute bioavailability of promethazine when applied in a PLO formulation was 2 %, and serum concentrations were much lower than after parenteral administration. A randomized, placebo-controlled, double-blind, crossover study of five volunteers, using 1 ml of morphine compounded at 10 mg/ml in a PLO for topical application, revealed that the bioavailability of morphine was unsatisfactory to provide cancer-related pain relief (Paice et al. 2008). Bleicher et al. (2008) investigated the efficacy of a PLO formulation containing ABH, i.e., lorazepam (Ativan) 2 mg, diphenhydramine (Benadryl) 25 mg, and haloperidol (Haldol) 2 mg, in reducing breakthrough chemotherapy-induced nausea and vomiting (CINV). Adults receiving standard recommended prophylactic antiemetics as outpatients were instructed to use 0.5 ml of the gel topically when they experienced significant CINV. Patients then were contacted retrospectively to respond to a questionnaire rating their nausea and/or vomiting and their response to ABH-gel treatment. The results were collected during two trials: Trial I began in April 2003, and Trial II began in March 2006. During Trial I, 23 patients were evaluated; 17 patients (74 %) reported that use of the gel decreased their CINV, with 15 (70 %) reporting relief within 30 min of its application. Three patients believed that the gel caused sedation; no troubles with skin irritation or muscle spasms were reported. In Trial II, all 10 patients believed that the treatment was effective. When the severity of CINV was quantified on a scale of 0–10, the mean CINV score decreased significantly from a 6.1 before gel application to a 1.7 as evaluated 30 min following gel application ($p < 0.005$). Topical use of ABH gel appears to be a promising and safe rescue therapy for breakthrough CINV that occurs despite prophylactic antiemetic therapy. These results warrant further confirmation in a large, randomized, placebo-controlled trial. Recently, Smith and associates tested the cutaneous absorption of ABH gel in healthy adults (Smith et al. 2011). They concluded that none of the lorazepam or haloperidol in ABH gel is absorbed in sufficient quantities to be effective in the treatment of nausea and vomiting. Benadryl (diphenhydramine) is absorbed at low levels, late, and erratically. Nevertheless, ABH topical gel is currently widely used in home hospice due to perceived efficacy and low cost. Very recently, Weiland et al. (2012) announced the clinical investigation on the transdermal absorption of chlorpromazine in PLO gel in healthy adults. The outcomes of the researches will help direct pharmacist recommendations for symptom management, thereby improving quality of life for patients.

17.4 Gelatin-Stabilized Microemulsion-Based Organogels

17.4.1 Composition and Formation of Gelatin-Stabilized MBGs

Gelatin-stabilized MBGs are transparent, viscoelastic, thermoreversible systems obtained by gelation of water-in-oil microemulsions with 10–20 % of gelatin (Atkinson et al. 1991; Haering and Luisi 1986; Kantaria et al. 2003; Quellet and Eicke 1986; Rees et al. 1998; Zhao et al. 2006). Similarly with LOs, the surfactant-to-water ratio is a crucial parameter for gelation phenomenon in gelatin-containing MBGs. Gelatin is a hydrophilic protein which forms a gelled structure when a heated solution is cooled down to a temperature below 35 °C.

Therefore, gelatin-stabilized MBGs are prepared by the addition of a gelatin powder to a water-in-oil microemulsion or a concentrated aqueous gelatin solution to a reverse micelle solution, at elevated temperatures, and then cooled down to room temperature (Haering and Luisi 1986; Quellet and Eicke 1986). The first preparation method is more preferred, because of thermostable nature and ease of preparation of the microemulsions (Santos et al. 2008).

Many studies have focused on the structure of MBGs (Atkinson et al. 1988; Atkinson et al. 1989, 1991; Białopiotrowicz and Janczuk 2002;

Caldararu et al. 1999; Petit et al. 1991). A gelation model describes the formation of the network of gelatin strands in water-continuous channels surrounded by an oil-continuous microemulsion. The hydrated gelatin network and water droplets are stabilized by an interfacial monolayer of surfactant. The introduction of gelatin had very little impact on the phase behavior of the microemulsion system. The viscosity of gelatin-containing MBGs is comparable with hydrogels (Kantaria et al. 1999).

Many of the articles on gelatin-containing MBGs for transdermal drug delivery are systems stabilized by anionic surfactant dioctyl sodium sulfosuccinate (AOT). Gelatin-based MBGs could be formed when using AOT as stabilizer with a wide variety of oils (with the exception of the medium and large triglyceride oils). In order to improve biocompatibility of such systems, Kantaria et al. (1999) attempted to produce alternative gelatin-containing MBGs formulated with pharmaceutically acceptable oils (e.g., IPM, IPP) and nonionic surfactants which are generally more acceptable. However, nonionic surfactants could not be used alone. Gelatin-containing MBGs were formulated by replacing ≤85 % of AOT with the nonionic surfactants such as Tween® 21, 81, and 85 (Kantaria et al. 1999, 2003). In a study of Zhao and coworkers (2006), gelatin-containing MBGs composed of IPM, AOT, Tween® 85, and water have been prepared, loaded with a model drug (butenafine hydrochloride), and characterized by rheological measurements and environmental scanning electron microscope (ESEM). Transparent and homogeneous MBGs could be formed when the concentration of gelatin in the selected water-in-oil microemulsion was in the range of 7.0–12.0 % w/w. MBGs became more compact with increasing the concentration of gelatin in the formulations. The addition of butenafine hydrochloride to the MBGs could weaken the interconnected network structures of the MBG systems. Figure 17.6 shows the effects of butenafine hydrochloride on the microstructures of MBGs.

Compared to the system free of butenafine hydrochloride (Fig. 17.6c), the interconnected network structures are looser. However, when the concentration of butenafine hydrochloride is 0.5 % w/w, the interconnected network structures are still remained. When the concentration of butenafine hydrochloride is 1.0 % w/w (Fig. 17.6f), much looser networks are formed, indicating that the interconnected networks of MBGs are gradually destroyed. By the visual observation, the MBGs turned into opaque. At the higher butenafine hydrochloride concentration, the addition of butenafine hydrochloride could weaken the network structures of MBGs, but the networks have not been destroyed completely.

17.4.2 Percutaneous Permeation Enhancement Potential of Gelatin-Stabilized MBGs

Gelatin-stabilized MBGs can be used to solubilize hydrophilic drugs as well as lipophilic substances (Kantaria et al. 1999, 2003; Zhao et al. 2006). Presence of conducting aqueous channels is related with the unique ability of such systems to conduct electricity which makes them attractive as potential carriers of hydrosoluble drugs for iontophoresis-enhanced transdermal delivery (Kantaria et al. 1999, 2003). The iontophoretic gelatin-stabilized MBGs cause higher release rates of the incorporated bioactive agent. That could increase the quantity of water-soluble drug delivered *via* transdermal administration route compared to passive diffusion. Kantaria et al. (1999) investigated iontophoretic transdermal delivery of the model water-soluble drug (sodium salicylate) from such vehicles. *In vitro* studies using adapted Franz diffusion cells were conducted to measure drug flux through split-thickness porcine skin. Under passive conditions, the drug flux from an aqueous drug solution (the control formulation) and from a drug-loaded gelatin-containing MBG was comparable, and the transdermal flux is proportional to the drug loading and to the iontophoretic current density. When an electrical current was applied, drug flux from the aqueous solution was greater than that from the MBG. This was explained by the fact that part of the drug entrapped within the MBG was present in the disperse aqueous droplets and

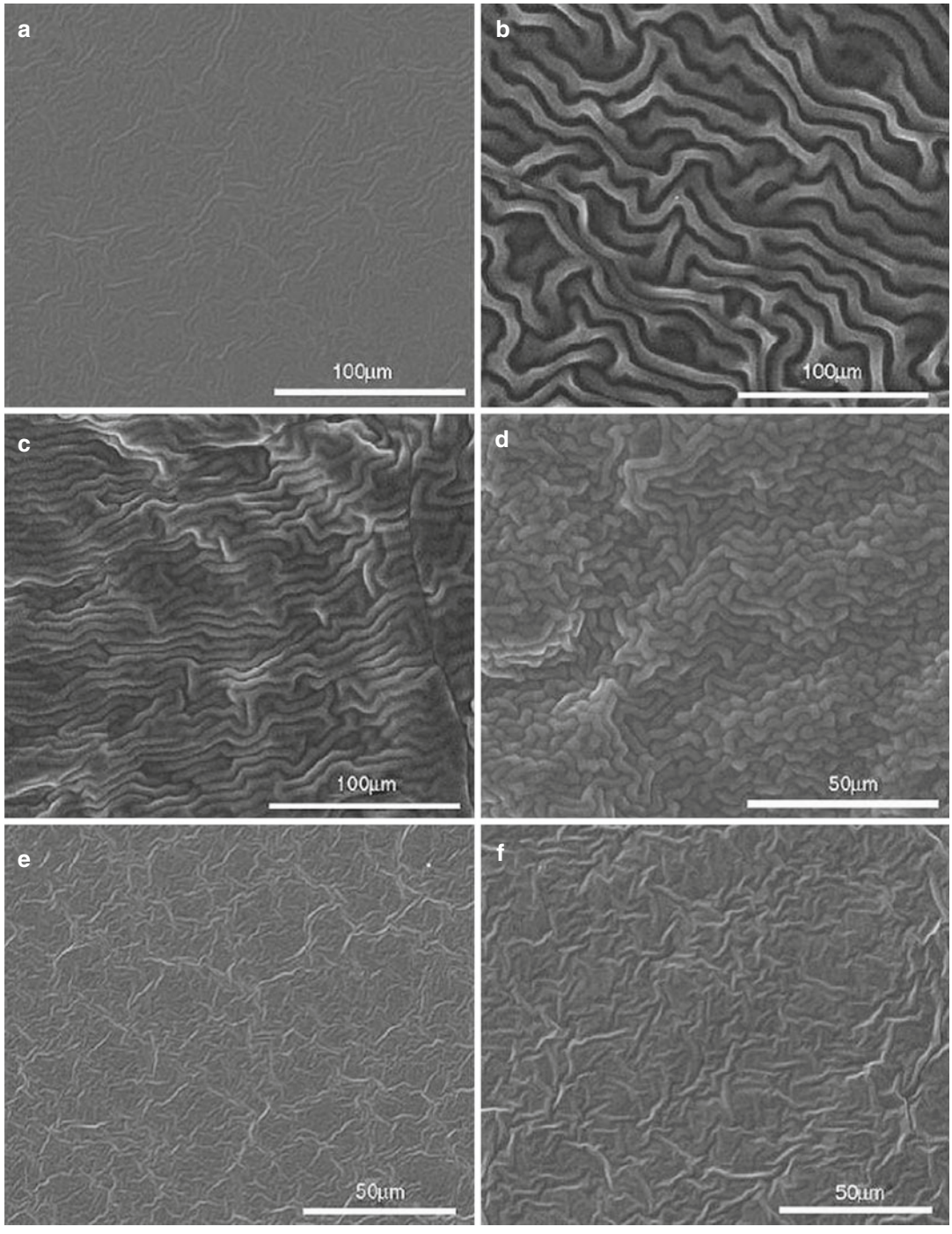

Fig. 17.6 ESEM images of the gelatin-containing MBGs, the concentration of gelatin: (**a**) 6.0 wt%, (**b**) 7.0 wt%, (**c**) 10.0 wt%, (**d**) 12.0 wt%. The concentration of butenafine hydrochloride: (**e**) 0.5 wt%, (**f**) 1.0 wt%, fixed the concentration of gelatin at 10.0 wt% (Zhao et al. 2006)

may not have been influenced by the applied electrical current. It was pointed that the advantage of the gelatin-containing MBG over the aqueous drug solution could be its higher viscosity, making it suitable for topical application, and its resistance to microbial contamination due to the large apolar oil component. Agrawal et al. (2004) investigated gelatin-containing MBGs as transdermal vehicles for piroxicam. The *in vitro* and *in vivo* permeation of the drug from gelatin-containing MBG comprising AOT/isooctane/water was compared with that from lecithin/IPM/water MBG and from a marketed formulation. Significant ($p<0.01$) inhibition of carrageenan-induced rat paw edema was observed for the investigated MBGs and a marketed transdermal product after 3 h. However, both MBGs were superior to the marketed product at inhibiting rat paw edema. Furthermore, *in vitro* as well as *in vivo* permeation of piroxicam from lecithin-based MBG was found to be superior to the gelatin-containing MBG.

In a recent attempt to develop gelatin-stabilized MBGs with enhanced safety, Xuan et al. (2012) introduced the formulation of lecithin-linker microemulsion-based gels (MBGs) prepared with gelatin as gelling agent. To produce these lecithin-linker microemulsions, it used lecithin as the main surfactant, sodium monooleate as a lipophilic additive (linker), and a mixture of PEG-6-caprylic/capric glycerides and decaglycerol monocaprylate/caprate as hydrophilic additives (linkers) that have been confirmed to be nonirritant to human skin, nonmutagenic, and highly biocompatible (Yuan et al. 2008). The gelatin was added to lecithin-linker microemulsion and a clear gel with a viscosity suitable for topical applications was obtained. The results suggest that some of the water phase of the parent microemulsion was used to produce a dispersed network of gelatin fibers embedded in an oil-rich bicontinuous microemulsion. This observation contrasts with previous findings for anionic (AOT) MBGs where the addition of gelatin produced minor changes in the morphology of the microemulsion. The lecithin-linker MBG enables similar release profile and slightly lower transdermal permeation of the model drug substance lidocaine, compared with the parent lecithin-linker microemulsion. It was concluded that the gelatin gel network did not affect the loading or release of drugs on the skin, but produce a viscosity suitable for topical application. Also, the skin-receiver transport coefficient values of the lecithin-linker MBG and the parent microemulsion were low. That was associated with the relatively high concentration of lecithin and hydrophilic linkers. Such lower values of the skin-receiver transport coefficient are desirable for extended drug release. Therefore, these formulations offered the potential for longer-lasting pain relief when compared to commercial lidocaine creams such as EMLA (emulsion containing 2.5 % w/w lidocaine and 2.5 % w/w prilocaine), whose action only lasts for 2–4 h (Yuan et al. 2008; Yuan 2009).

17.5 Summary

MBGs are systems with the unique characteristics including their gelation behavior, thermodynamic stability, viscoelasticity, and thermoreversibility. Interest on use of the MBGs as drug delivery vehicles for dermal and transdermal drug delivery has grown in the last decade. The clear advantages are their biocompatibility, ease of preparation and administration, and increased potential for improvement of percutaneous penetration and effectiveness of the topically applied drugs. Ready-to-use lecithin-based MBG vehicles for pharmacy compounding are already in use, although the doses of the drugs which are applied transdermally are quite empirical. More studies should be done in order to further elucidate the value of MBGs as topical and transdermal delivery vehicles.

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Nanocrystals for Passive Dermal Penetration Enhancement

18

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18.1 Introduction: What Are Nanocrystals?

January 25, 1991 can be seen as the "date of birth" of the pharmaceutical nanocrystals, and it is the filing date of the patent submission by Liversidge and coworkers (1992). They claimed crystalline drug nanoparticles (= nanocrystals) with a size below 400 nm having an increased oral bioavailability. The particles consist of 100 % of drugs and are typically stabilized by a surrounding surfactant or polymer layer. In contrast to matrix nanoparticles, there is no matrix material, thus yielding maximum drug loading. The drug loading is distinctly superior to matrix nanoparticles having drug adsorbed to the surface or incorporated inside the matrix (Fig. 18.1). Nanocrystals are in most cases produced in liquid media, and the resulting products are nanocrystals

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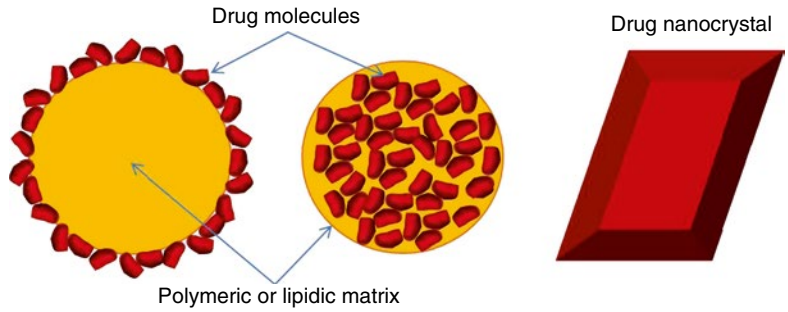

Fig. 18.1 Structure of polymeric or lipidic matrix nanoparticles having drug adsorbed on the surface (*left*) or incorporated inside the matrix (*middle*) versus nanocrystals (*right*). The drug loading increases from left to right, being 100 % for the nanocrystals

dispersed in a liquid, i.e., the nanosuspensions. The liquid is typically water (aqueous nanosuspensions) which contains a stabilizing surfactant (e.g., polysorbate (Tween®, Sigma-Aldrich, Germany)), polyhydroxy glucosides (e.g., Plantacare® series, Cognis, Germany, etc.) or a sterically stabilizing polymer (e.g., block copolymers (Poloxamer® series, BASF, Germany), polyvinyl alcohol, xanthan, etc.). However, there are also nonaqueous nanosuspensions described, e.g., with liquid polyethylene glycol (PEG) or oils (e.g., caprylic/capric triglycerides, i.e., Miglyol®, Sasol, Germany) (Müller et al. 2000).

Based on the pharmaceutical definition of nanoparticles (Müller et al. 2011a), the size of drug nanocrystals ranges from a few nm to 1000 nm (=1 µm), being the "border" to the microparticles. According to the regulatory authorities, dermal cosmetic products need to be labeled as "nano products" when they contain particles below a size of 100 nm, which seems somehow contradictory. Also the US Food and Drug Administration (US FDA) is using this size limit (US FDA 2011). The reason for this size limit is that these very small nanoparticles <100 nm have potentially (but not necessarily!!) a higher toxicity risk than nanoparticles >100 to 1000 nm (cf. Sect. 18.8). However, the basis for the size range 1–1000 nm is the definition of nanotechnology: nanotechnology creates new physicochemical properties by moving the size of materials to the nano range. The size at which these changes occur is approximately 1 µm = 1000 nm. Below this size many physical properties change, e.g., as with nanocrystals the saturation solubility, the color, and the adhesiveness.

18.2 Nanocrystals in Marketed Products

In general cosmetic industry watches carefully innovative developments in delivery technology in pharmaceutical industry and applies it to its cosmetic products. The classical example is the use of liposomes. They were described by Bangham in the middle of the 1960s (Horne et al. 1963; Bangham and Horne 1964; Bangham 1963, 1972, 1978), and pharmaceutical development was going on, but the first product on the market was the cosmetic antiaging gel "Capture" launched by Dior in 1986 (Calistro 1987; Lasic 1995). The first pharmaceutical products appeared on the market around 1990, e.g., Alveofact® by Dr. Karl Thomae GmbH, now Boehringer Ingelheim, and liposomes for dermal application Epi-Pevaryl® containing econazole, launched by Cilag in 1988. In case of the nanocrystals, the cosmetic industry missed for many years the opportunity. Nanocrystals can enhance the drug penetration into the gut wall, but of course they can also increase the drug penetration into the skin, based on exactly the same physical principles.

The first nanocrystal pharmaceutical product appeared on the market in the year 2000 (Rapamune® by the company Wyeth), while cosmetic industry was late. The first dermal products with rutin nanocrystals were Juvedical® Renewing Serum and Juvedical® DNA Skin Optimizer Fluid and Cream SPF 20 by Juvena, Switzerland in 2007. They exploited the passive penetration-enhancing effect of the nanocrystals to make the rutin powder highly active in the

skin, which was not possible when using micronized rutin. This chapter describes the special physical features of nanocrystals leading to passive enhancement of drug penetration and explains the mechanism, but also provides information about the incorporation of nanocrystals into final dermal cosmetic and pharmaceutical products. Also the increasing concern about nanotoxicity is addressed for the nanocrystals, placing them into the nanotoxicological classification system (NCS).

18.3 Special Features of Nanocrystals for Dermal Delivery

18.3.1 Solubility Properties

Nanocrystals, like other nanosized materials, have in general a high surface area to volume ratio and a large number of surface-located molecules in contact with the dispersion medium. Compared to the micro-sized particles, some of the physicochemical properties of the particles change when they are transferred into the nanodimension. Examples of these changes are:

- Improved solubility properties (saturation solubility c_s, dissolution velocity dc/dt)
- Increase in adhesiveness to surfaces
- Decrease in melting temperature
- Different optical and magnetic properties

In special, the solubility property changes of nanocrystals (Buckton and Beezer 1992) are of great interest regarding active ingredient penetration into and permeation through the skin. The saturation solubility c_s increases for sizes of below approx. 1 μm due to an increased dissolution pressure (Kelvin equation), and the dissolution velocity increases due to both increase of c_s and surface area A of the particles (Noyes-Whitney equation). This improved solubility behavior is the main feature exploited when using nanocrystals in dermal formulations. Passive diffusion is by far the most common way of penetration of active ingredients into the skin. The nanocrystals enhance this passive diffusion by increasing the concentration gradient between the dermal formulation and the skin. The dissolved drug diffused from the dermal formulation into the skin, and it will be immediately replaced by the fast dissolving drug from the nanocrystals in the dermal formulation (= drug depot) (Fig. 18.2, upper).

Fick's law of diffusion defines the flux of chemicals permeating through the stratum corneum, J, as

$$J = k_p \cdot \Delta C, \qquad \text{where} \quad k_p = \frac{K \cdot D}{L}$$

k_p–permeability coefficient
ΔC–concentration gradient over the stratum corneum
K–octanol/water partition coefficient
D–diffusivity
L–length of the diffusion pathway of the permeating molecule

From the formula one can imply that in order to achieve the highest permeation rate, the concentration gradient ΔC over the stratum corneum should be as high as possible. Due to the increased saturation solubility c_s, nanocrystals offer the possibility to achieve a higher concentration of free molecules of the drug/active dissolved in the water phase of the formulation when compared to standard formulations. As a consequence, there is an increased concentration gradient of the active between the top and the bottom layers of the stratum corneum (ΔC). This increases the passive diffusion through the stratum corneum and subsequently to the viable layers of the epidermis.

18.3.2 Size Effects

The skin being such an effective barrier, it is very unlikely that intact nanoparticles could penetrate in a substantial number deeply into it or even permeate through it. Improved penetration was reported for dendritic nanoparticles of around 40 nm (Küchler et al. 2009), but the normally applied larger-sized carriers have very little access. Therefore enhancing the passive diffusion by nanocrystals is a much smarter approach than

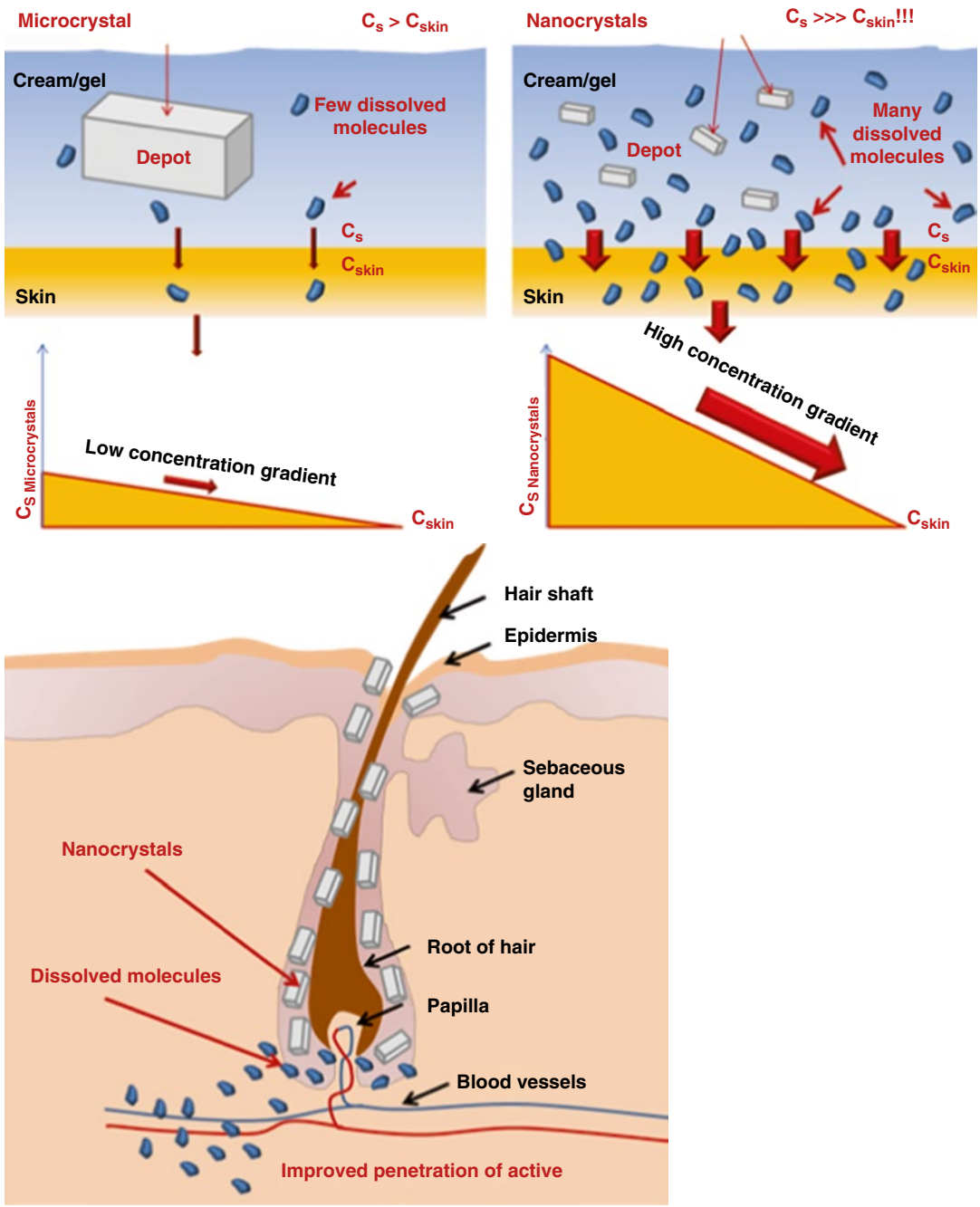

Fig. 18.2 Delivery mechanisms of nanocrystals on the skin: *Upper*, in contrast to microcrystals nanocrystals create a higher saturation solubility, thus increased concentration gradient. Penetrated drug is replaced by molecules dissolving from the depot nanocrystals (Modified after Keck and Müller 2013a). *Lower*, nanocrystals accumulate in the hair follicles, create locally supersaturation of the active with increased penetration of the active into surrounding skin

trying to get the drug into the skin by penetrating loaded in nanoparticles. Of course, nanocrystals can also be made in sizes around 50 nm or below (e.g., amphotericin B). Production methods are combination technologies, e.g., precipitation and subsequent high-pressure homogenization (HPH) (Müller et al. 2007) or lyophilization and HPH (Möschwitzer et al. 2007). However, these pro-

cesses are more costly than standard nanocrystal production by HPH alone or by bead milling, thus limiting their use to more expensive drugs and unmet needs.

Lademann et al. (2001, 2005, 2007) reported that nanoparticles can localize in the hair follicles. This localization leads to higher drug concentration in the follicles compared to applying a solution, and optimal nanoparticle size for this effect is around 650 nm (measured by photon correlation spectroscopy) (Patzelt et al. 2011). In that investigation polymeric model nanoparticles were used. The drug accumulation in the hair follicles could be further increased by replacing polymeric nanoparticles with low-to-medium drug load by nanocrystals with 100 % drug load. The nanocrystals lead in addition to a higher solubility of the drug in the hair follicle, promoting further the diffusion into the surrounding skin (Fig. 18.2, lower).

18.3.3 Adhesion

Identical to all nanosized materials, nanocrystals are adhesive to surfaces (Xing et al. 2010; Fernandez 2007). This can be explained in a simplified way. One large crystal of 10 µm (assuming shape of a cube) has a surface of attachment of $10 \times 10 = 100$ µm^2. Disintegrating this cube yields, for example, 1000 cubes of 1 µm, which have a surface of attachment of 1000×1 µm^2 $= 1000$ µm^2, i.e., 10 times more. Diminution of the micrometer cube particle to 0.1 µm nanocrystals yields 100 times more surface for contact. With the size of the attachment area, the interactions increase. From this, the nanocrystals remain adhered to the skin after evaporation of the water phase of the dermal formulation, much better than micronized powders, possessing a much smaller contact area.

18.4 Production Technologies of Nanocrystals

Nanocrystals can be produced by bottom-up and by top-down technologies; for details it is referred to (Müller et al. 2011b; Keck and Müller 2006). In the bottom-up technologies, one starts from single molecules, and let them aggregate and grow to crystals, i.e., it is the classical precipitation. Sucker developed precipitated nanocrystals which he called hydrosols (Gassmann et al. 1994; List and Sucker 1988). A solvent with the dissolved drug is added to a miscible non-solvent, and crystalline nanocrystals precipitate. In a modified precipitation process by Auweter from the company BASF (Germany), amorphous drug nanoparticles are formed in the precipitation process (Auweter et al. 2002). This process is called NanoMorph technology for pharmaceutical products (company Soliqs, Germany). In the pharmaceutical area, to our knowledge no products based on these processes are on the market. Reasons are manifold, e.g., the precipitation process is difficult to control, and organic solvents need costly to be removed. Amorphous drug nanoparticles are superior in solubility enhancement, but the industry is afraid that they might recrystallize during the shelf life of a product leading to a decrease in bioavailability. Therefore if possible the industry tries to solve delivery problems with crystalline drug nanoparticles (= nanocrystals) to be on the safe side, definitely the products with NanoMorph will come. Amorphous nanoparticles are on the nutrition market launched by BASF, i.e., carotenoids for addition to soft drinks (e.g., product Lucarotin®).

The present nanocrystals in dermal cosmetic and in pharmaceutical products are made up by top-down technologies, i.e., one starts from a larger crystal and reduces it in size by various wet milling processes. The most important milling processes are:

One step processes:
Bead mills for wet milling (by ALZA, USA, previously élan/Nanosystems, USA) (Liversidge et al. 1992)
High-pressure homogenization (HPH) (SkyePharma PLC, UK, prev. dds GmbH, Germany) (Müller et al. 1999)
Combination technologies:
Precipitation plus subsequent HPH (NANOEDGE, Baxter Inc USA) (Kipp et al. 2003, 2005)
Various combination technologies[a]

[a]The preferred combination technology is bead milling plus subsequent HPH Petersen (2006), the so-called CT process (Abbott/Soliqs, Germany, prev. PharmaSol GmbH Berlin). The trade name is smartCrystal®.

18.5 Industrial Large-Scale Production of Nanocrystals for Dermal Market

All the top-down technologies have the advantage that they can be run on small lab scale (batch size 5–40 g), on intermediate scale (0.5 – a few kg), and on large scale (a few 100 kg suspension). For example, high-pressure homogenizers can be purchased with increasing capacity, e.g., Avestin B3 (approx. 5 ml, Avestin), Micron LAB 40 (20–40 ml, APV Gaulin) or Panda 2 K (3–10 l/h, Niro Soavi), Avestin EmulsiFlex-C50 (50 l/h) or C1000 (1000 l/h, all Avestin). Also bead mills are available from very small to large scale (e.g., Dyno-Mill by Bachofen, PML 2 by Bühler, etc.). Most of the pharmaceutical products on the market contain nanocrystals produced by bead milling (Rapamune®, Emend®, Tricor®, Megace ES®, INVEGA SUSTENNA®), one product is produced by high-pressure homogenization (Triglide®, SkyePharma technology).

Nanocrystals in cosmetic products are primarily produced by the combination technology (CT) process. Concentrates can be purchased which are admixed to the outer phase (water) of ready produced o/w creams or o/w lotions. Available are, e.g., rutin (INCI: rutin submicron) and hesperidin (INCI: smartCrystal-lemon extract) and various specially made products. Producer is the company Dr. Rimpler GmbH in Wedemark/Hannover, Germany (www.rimpler.de).

18.6 Incorporation of Nanocrystals into Dermal Formulations

The incorporation of nanocrystals into dermal products is a very simple process. The dermal formulation (e.g., o/w cream or lotion) is produced with a slightly reduced water content, e.g., taking out 2 % of water (instead 100 % formulation weight, this results in 98 % weight). During the cooling process of the product, close to room temperature, a 5 % nanocrystal concentrate is added (e.g., 2 kg concentrate to 98 kg product, dilution factor = 50) and admixed applying low shear mixing. A part of the nanocrystals will dissolve until the saturation solubility c_s is reached. This nanocrystal solubility (kinetic solubility) is higher than the thermodynamic solubility of the drug/active. A part of the nanocrystals will remain non-dissolved; these remaining nanocrystals will act as depot in the cream (c.f. Fig. 18.2, upper).

It is important that the concentration of nanocrystals in the product is higher than their solubility. Otherwise no nanocrystals will remain as depot, and the kinetic supersaturation might not be long-lasting. As a rule of thumb, a concentration five times higher than the kinetic c_s is recommended for actives poorly soluble in water and simultaneously in the oils of the formulations. For oil-soluble actives, the solubility in the oil needs to be considered. The oil phase acts as acceptor medium, and with a long storage time, active from the nanocrystals in the water phase will diffuse via the water into the oil phase. This effect can be avoided by making gel formulations.

18.7 Nanocrystals of Poorly Soluble Actives and Marketed Products

Formulation challenges are actives which are poorly soluble in water and simultaneously in lipophilic media, e.g., oils. Nanocrystals are the formulation principle for these poorly soluble actives, which do not show sufficient penetration into the skin when applied as microcrystals in a dermal formulation (e.g., micronized powder). This is valid for many natural plant compounds and pure compounds but also poorly water-soluble extracts. Typical examples are flavonoids, e.g., rutin, hesperidin, hesperitin, or resveratrol. The saturation solubility of these compounds in the water phase of the dermal formulations is low, providing a too low concentration gradient for efficient penetration. In addition, penetrated actives are only slowly replaced by new molecules dissolving from the micrometer-sized crystals (cf. Fig. 18.2, lower).

This was nicely shown in a study in humans comparing dermal formulations containing rutin

Fig. 18.3 Commercial products with hesperidin and rutin nanocrystals. *Left*: platinum rare by la prairie (Switzerland), hesperidin nanocrystals. *Right*: ageLine wo/man one, Eye Lifting Serum with rutin nanocrystals (*red arrow*) by ipam/Berlin (by permission of ipam). Due to the size of the rutin nanocrystals being >>100 nm, these products are no classified cosmetic nanoproducts (Color figure online)

as a water-soluble derivative (rutin-glucoside) versus rutin nanocrystals. The formulations contained 5.0 % rutin-glucoside versus about 0.01 % dissolved rutin in the nanocrystal formulation (plus nanocrystal depot), i.e., 500 times lower dissolved active. The antioxidant effect was quantified by measuring the sun protection factor (SPF) in human subjects after irradiation. The SPF increases with increasing antioxidant effect. The nanocrystal formulation showed about double increase in SPF at 500 times lower dissolved active, i.e., in a simplified way: about 1000-fold increase in biological activity (bioactivity) (Petersen 2006). The mechanism of action is explained by three factors:

1. High concentration gradient of nanonized rutin, immediate replacement of penetrated rutin from dissolving nanocrystal depot = maintaining concentration gradient
2. Better penetration of more lipophilic rutin into the skin compared to the very hydrophilic rutin-glucoside (preferred to stay in hydrophilic water phase of formulation)
3. Higher biological activity of original rutin in the cells compared to synthetic derivative

A very good antioxidant activity was also found for hesperidin. Both flavonoids are in marketed products (Fig. 18.3).

To clarify the mechanisms, also various in vitro studies were performed (Petersen 2006; Chen et al. 2013; Sinambela et al. 2012). The increase in antioxidant capacity (AOC) of nanonized antioxidants can be shown in in vitro test systems. At present one of the most efficient test methods to predict the total AOC efficiently and with a strong relation to the in vivo performance is the full name (KRL)-test (Chen et al. 2013; Blache et al. 2006 developed by Prost 1989, 1992). The test measures the resistance of red blood cells against the free radicals induced by 2 2'-azobis (2-amidinopropane) hydrochloride (AAPH). The resistance of whole red blood cells to free radical attack is expressed by the time that is required to reach 50 % of the maximal hemolysis ($HT_{½}$). Hence, $HT_{½}$ will increase with increasing AOC of the sample. The increase in $HT_{½}$ was

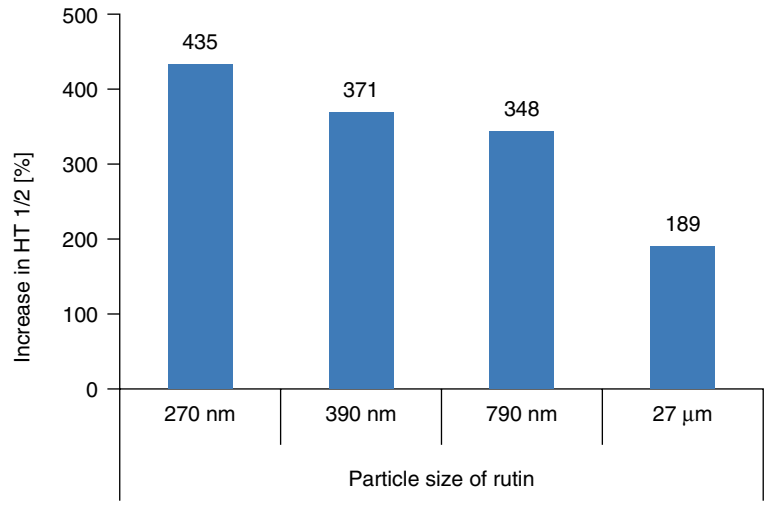

Fig. 18.4 Increase in antioxidant capacity (expressed in half time hemolysis ($HT_{½}$)) of differently sized rutin crystals, from 27 µm microcrystals to nanocrystals of decreasing size (Modified after Chen et al. 2013)

tested for rutin bulk material (mean size about 27 µm) and for rutin nanocrystals with sizes of 790 nm, 390 nm, and 270 nm, respectively. The increase in $HT_{½}$ was about 190-fold for the bulk material when compared to the control proving the antioxidant activity of the rutin. The $HT_{½}$ was about 350-fold and 370-fold for the nanoparticles possessing a size of 790 and 390 nm. The increase in $HT_{½}$ was 435-fold for the nanoparticles with a size of about 270 nm, i.e., the AOC of these particles is more than 2-fold higher when compared to the rutin bulk material (Chen et al. 2013, Fig. 18.4).

18.8 Nanocrystals of Poorly Soluble, Lipophilic Actives

Actives which are poorly water soluble, but soluble in organic media, are typically incorporated in creams and lotions by dissolving them in the oil phase, e.g., vitamin E or coenzyme Q10 (Q10). The question is: provide nanocrystals also formulation advantages for these lipophilic oil-soluble actives?

The penetration mechanism of the oil-soluble compounds is:

1. Partitioning from the oil to the water phase of the cream (according to log *P*, Nernst partitioning coefficient *K*)
2. Presence of a low concentration of active in the water phase due to the low water solubility
3. Diffusion from water phase into the stratum corneum/skin (Fig. 18.2, upper)

In the case of, e.g., a Q10 nanosuspension as gel, the partitioning step will be omitted, the concentration of dissolved active in the water phase is higher; thus, a higher concentration gradient results promoting increased penetration. From this it makes sense to formulate oil-soluble actives in oil-free dermal formulations (gels) as nanocrystals.

18.9 Nanocrystals of Water-Soluble Actives: The Novel Approach

At the first glance, preparation of nanocrystals from a water-soluble active appears nonsense. It is already soluble, why nanocrystals? However, in some cases this makes absolute sense, e.g., for caffeine. Caffeine is increasingly used in anticellulite products. The trend is to put products on the market with increasing caffeine concentration, which is limited by the water solubility, being about 4 % at room temperature (strongly temperature dependent!). In penetration studies it was found that the caffeine concentration in the

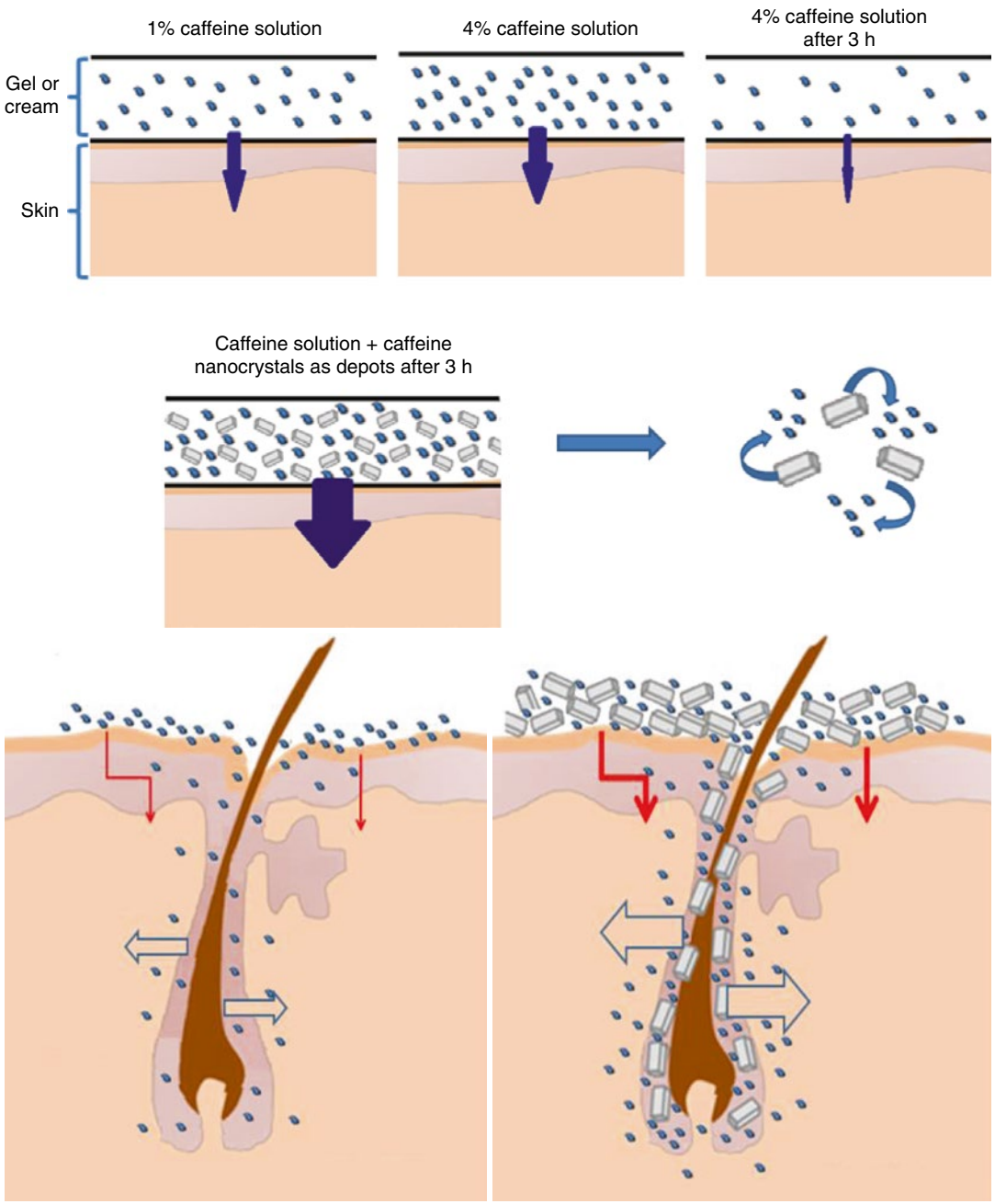

Fig. 18.5 Theory of increase of penetration of caffeine by the use of a caffeine nanocrystal nanosuspension: the caffeine concentration in the formulation (caffeine solution) decreases due to penetration (*upper*). Increased penetration into the skin occurs by maintaining a supersaturated caffeine solution on the skin by the nanocrystals present (*middle*), and – compared to caffeine solution on skin (*lower left*) – higher caffeine concentrations in the skin can be reached by localization of nanocrystals in the hair follicle (*lower right*)

stratum corneum increases with increasing caffeine concentration in the applied formulation (unpublished data). In a dermal formulation with maximum caffeine dissolved, the caffeine concentration decreases with time due to penetration into the skin (Fig. 18.5, upper). Having nanocrystals present, they act as fast dissolving depot, keeping the concentration constant (Fig. 18.5,

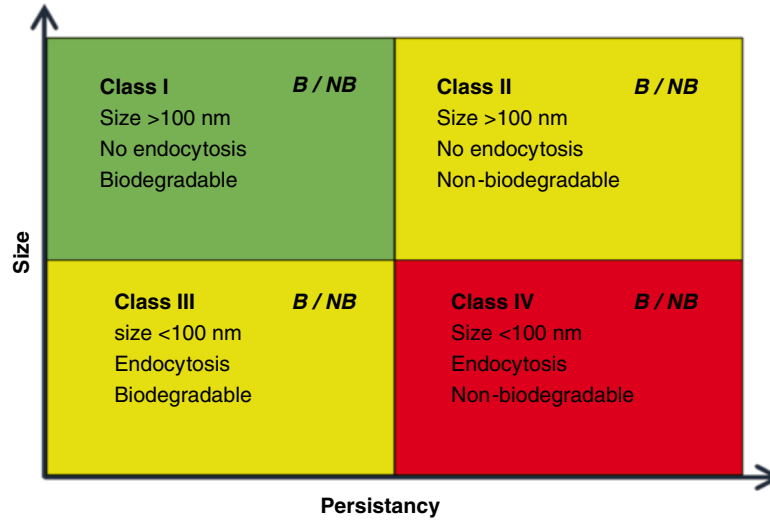

Fig. 18.6 Nanotoxicological Classification System (NCS) classifying the nanoparticles in class I (>100 nm, biodegradable), class II, (>100 nm, nonbiodegradable), class III (<100 nm, biodegradable), and class IV (<100 nm, nonbiodegradable), in traffic light system from *green* to *yellow*. Each class is subdivided in B (biocompatible) and NB (non-biocompatible) (Modified after Müller et al. 2011a)

upper). Even more important, the caffeine nanocrystals can localize in the hair follicles, creating here an increased concentration gradient and depot for diffusion into the surrounding skin cells (Fig. 18.5, lower).

18.10 Nanotoxicity/ Nanotolerability of Nanocrystals

Potential toxicity of nanoparticles is an increasing issue, not only in the USA but also in European countries. This raises also the question: How do we judge the nanocrystals?

The legal authorities give a lot of attention to ensure the safety of nanoproducts. In the future, there will be increased analytics to be performed by producers of nanoparticles as intermediate products and producers of final market products containing nanoparticles. First problem, the analytical requirements are still unclear, which methods yield really a result relevant to nanotoxicity. Secondly, the number of nanoparticle products is meanwhile huge in many different fields, from nanopolish for cars, via food, to cosmetics and pharma products. The problem is which nanoparticles have the most urgent need to be evaluated first regarding potential nanotoxicity, because the analytical/financial resources are limited.

To tackle with this problem, a system is needed to classify the nanoparticles regarding potential risk, from potentially no/low risk to potentially high risk, allowing focusing resources on the most risky nanosystems first. In addition such a system would allow the consumer better assessment of the products he/she is buying/using. Analogous to the Biopharmaceutical Classification System (BCS) by Amidon et al. (1995), recently the Nanotoxicological Classification System (NCS) was proposed (Keck and Müller 2013b).

The NCS includes nanoparticles from 1 to 1000 nm. It classifies the nanoparticles in classes according to size (≤100 nm, >100–1000 nm) and according to their biodegradability in the body (biodegradable, nonbiodegradable), yielding four classes I to IV. In addition each class is specified as being a biocompatible (B) nanoparticle or a non-biocompatible (NB) one. This yields a total of 8 differentiations: I-B, I-NB, II-B, II-NB, III-B, III-NB, IV-B, and IV-NB. The NCS is depicted in Fig. 18.6.

What is the rationale behind the NCS? As outlined in Sect. 18.1, the European authorities define particles as nanoparticles only when their size is below 100 nm. Precisely (European Commission Recommendation 2011):

- "Nanomaterial" means a natural, incidental, or manufactured material-containing parti-

cles, in an unbound state or as an aggregate or as an agglomerate and where, for 50 % or more of the particles in the number size distribution, one or more external dimensions is in the size range 1–100 nm.
- In specific cases and where warranted by concerns for the environment, health, safety, or competitiveness, the number size distribution threshold of 50 % may be replaced by a threshold between 1 and 50 %.

Simplified: the 100 nm threshold was selected because such small-sized material can enter any cell by endocytic processes. Particles larger than 100 nm can only enter cells which have a phagocytic mechanism, e.g., liver macrophages. Due to the limited number of cells which these particles can access, and the difficulty to access just these cells at all (e.g., macrophages in the liver), these nanoparticles possess a lower risk. Of course the 100 nm is a legal definition and scientifically speaking not that strict/correct. There are some mechanisms also allowing uptake of particles >100 nm by non-phagocytic processes. However to make a law or guideline enforceable, a definite measurable size needs to be given. Consequently the nanoparticles >100 nm possess a lower risk, reflected in classifying them in class I and II (Fig. 18.6).

If particles are biodegradable, they will eventually disappear in the body, opening the perspective that also the caused side effect disappears. Therefore they are a priori less risky than the nonbiodegradable ones which stay forever in the body (e.g., fullerenes). It is important to highlight that the NCS indicates only potential risk/no risk! Even biodegradable particles can cause negative effects during their time of existence, e.g., irritating the immune system! On the other hand, nonbiodegradable <100 nm nanoparticles are not necessarily toxic, e.g., radioactive gold and antimony sulfide nanoparticles are used as IV diagnostics for gamma scintigraphy.

Nanocrystals are clearly classified as well-tolerated class I particles. When smaller nanocrystals are used (<100 nm), they belong to class II. Of course during their dissolution process, the nanocrystals will shrink below 100 nm, thus turning into class II. Based on this, the nanocrystals are a well-tolerated delivery system to the skin, as far they are not made from skin-irritating or skin-toxic chemicals! Of course chemical toxicity dominates physical tolerability according to the NCS. The tolerability of nanocrystals is also documented, i.e., that the nanocrystals are accepted in oral pharmaceutical products since the year 2000.

18.11 Conclusions and Perspectives

Besides the liposomes, the nanocrystals are one of the most successful nanosystems ever developed in pharmaceutical industry. Time from invention to the market was about 25 years for the liposomes (approx. 1965–1990), only 9 years for the nanocrystals (1991–2000). Products on the pharmaceutical market include also blockbusters (e.g., Tricor® from Abbott).

The principle of action of the nanocrystals is simple; it is all physics, and physics is reproducible and (to certain extent) controllable. For delivering the drug/cosmetic active, it is not necessary that the particle itself penetrates a membrane or permeates. This makes the nanocrystals clearly advantageous compared to, e.g., transfersomes (Cevc 1996). Delivery to the skin takes place via simple supersaturation and thus increasing the passive diffusion. Degree of supersaturation can be controlled by the crystal size, and it increases with decreasing size. Thus most effective dermal nanocrystals will be the ones with a size of about 20–50 nm.

The make ability of dermal nanocrystal products is proven by the cosmetic products on the market, the dermal efficiency proven by the in vivo studies performed with them (e.g., Petersen 2006; Müller et al. 2007; Chen 2013). Further cosmetic products will come, because there is a commercial supply of nanocrystals on industrial scale – essential prerequisite for products.

However, by now the delivery potential of nanocrystals has not yet really been exploited in pharmaceutical industry. What are the reasons for

this? They might be multifactorial. Dermal products are sometimes considered as niche market, and blockbusters are more likely in other areas. In addition, when a company has a selling traditional dermal product, one will not necessarily replace it with a more efficient but costly new development. Also company policies changed in view of financial crises, with concentration on traditional business and less risky new developments or investments. Despite this, nanocrystal products are expected on the market to formulate drugs, which are only active in a nano form. Such unique products, introducing new actives to the market via nanotechnology, can be enabled via the nanocrystals. Yes, we can make dermal pharmaceutical nanocrystal products.

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Vesicles for Transdermal Delivery of Peptides and Proteins

19

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Contents

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19.1 Introduction

19.1.1 Vesicles as Delivery Systems for the Skin

Vesicles are colloidal systems formed due to the tendency of amphiphilic molecules to self-aggregate into spherical structures in an aqueous solution. In the case of liposomes, discovered by Bangham in 1963, the amphiphilic molecules are generally phospholipid. The vesicles can be unilamellar or multilamellar depending on the nature of the phospholipid and method of production. They are capable of delivering both hydrophilic substances in their inner core and lipophilic substances in the lipid bilayer. These phospholipid-based liposomes are termed 'conventional' liposomes or vesicles in this chapter to distinguish them from the wide range of vesicles developed over the past 40 years. In the 1970s, L'Oreal et al. (1975) patented nonionic surfactant vesicles termed niosomes for the enhanced skin deposition of cosmetic agents. Cevc and Blume (1992) introduced the first generation of elastic or deformable vesicles termed Transfersomes. This has been followed by the development of many exotically named elastic vesicles of various compositions such as ethosomes (Touitou et al. 2000), invasomes (Dragicevic-Curic et al. 2008), and rovisomes (Blume et al. 2000). It is these elastic vesicles that are of most interest in the transdermal delivery of peptides and proteins.

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19.2 Conventional Liposomes

Mezei and Gulasekharam (1980) described higher skin deposition and reduced urinary excretion of triamcinolone acetonide from a liposomal formulation, compared to a control lotion in rabbits. A number of reviews (Elsayed et al. 2007; Cosco et al. 2008; Sinico and Fadda 2009) have summarised the research on conventional liposomes, showing that the most commonly encapsulated drugs are corticosteroids, anaesthetics, vitamins and retinoids. These reviews provide an excellent summary of the research in vesicle delivery of small molecules, whilst this chapter is focussed on the application of vesicles for peptide and protein delivery. The property of increasing drug deposition within the skin whilst reducing penetration through the skin is a commonly reported feature of conventional liposomes. Despite some early reports to the contrary, the general consensus is that conventional liposomes do not permeate the skin intact. They have been shown to accumulate in skin appendages, possibly offering an opportunity for targeting these sites in appropriate applications such as acne and hair growth (Jung et al. 2006; Tabbakhian et al. 2006).

Niosomes are vesicles composed of nonionic surfactants with cholesterol or other lipids. They have been evaluated as carriers for a number of cosmetic and drug applications. They are nontoxic and provide advantages over liposomes with regard to lower production cost and better stability over a longer period of time in different conditions. Mahale et al. (2012) provide an excellent review of the formulation, preparation methods, characterisation, and applications of niosomes. Surfactants are known to enhance skin penetration by disruption of the stratum corneum lipids. It has been suggested that niosomes, vesicles that are composed of surfactant molecules with a highly polar moiety, may disorder the stratum corneum lipid bilayers to facilitate drug permeation (Hofland et al. 1995). Thus, the delivery mechanism may be a combination of the penetration enhancement effect of the surfactant in the skin and the encapsulation of the drug within the vesicle structure. Although there is some evidence that niosomes may provide a higher initial flux and shorter lag time compared to conventional phospholipid-based liposomes, they exhibit a similar overall permeation rate (Carafa et al. 2002). In their examination of the interaction of vesicles with the stratum corneum lipid domains, Bouwstra et al. concluded that the mechanism of skin permeation of niosomes and phospholipid-based vesicles is similar (Bouwstra et al. 2003). It is therefore likely that the main application of niosomes is similar to conventional liposomes, that is, localised accumulation in superficial skin layers or follicular delivery for cosmetic and dermatological applications. Given their localisation within the stratum corneum and appendages, these niosome vesicle systems do not have potential for the transdermal delivery of peptides and proteins. However, as discussed later in this chapter, nonionic surfactants may be components of elastic vesicles, which have been utilised for peptide delivery.

19.3 Elastic or Highly Deformable Vesicles

A new class of liposomes termed Transfersomes® was first described by Gregor Cevc (Cevc and Blume 1992) and has been the subject of numerous patents and literature reports. A recent review on the topic provides an excellent background to the literature on Transfersomes (Kumar et al. 2012). Transfersomes belong to the category variously termed deformable, highly deformable, elastic or ultra flexible liposomes or vesicles. Elastic vesicles are claimed to permeate as intact vesicles through the skin layers to the systemic circulation and have been reported to improve in vitro skin delivery (El Maghraby et al. 1999; Cevc and Blume 2001; Trotta et al. 2004) and in vivo penetration (including peptides and proteins), to achieve therapeutic amounts comparable with subcutaneous injection (Cevc 2003). Transfersomes are composed mainly of phospholipids but also contain surfactant such as sodium cholate, sodium deoxycholate, Spans, Tweens and dipotassium glycyrrhizinate (El Maghraby et al. 1999; Boinpally et al. 2003; Trotta et al.

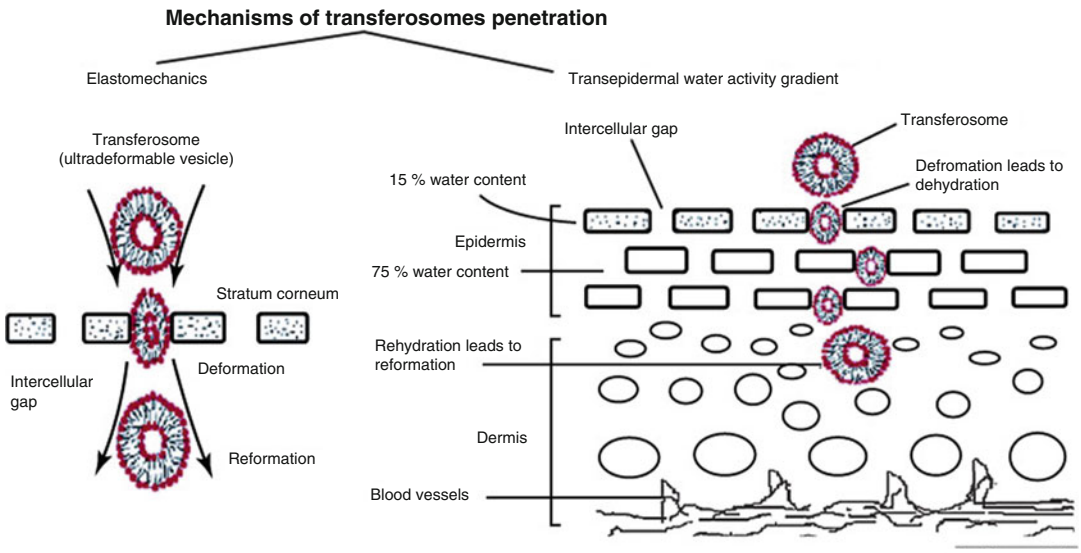

Fig. 19.1 Mechanisms of penetration of Transfersomes through the skin (Reproduced from Kumar et al. (2012) with permission from Elsevier)

2004). The surfactant acts as an 'edge activator' that destabilises the lipid bilayers and increases deformability of the vesicle (Bouwstra et al. 2002; Honeywell-Nguyen et al. 2004). The formulation may also contain some ethanol (typically up to 10 %) and a total lipid concentration of up to 10 % in the final aqueous lipid suspension (Cevc et al. 1995; Cevc 1996).

Due to the flexibility conferred on the vesicles by the surfactant molecules, Cevc (1996) reported that Transfersomes are able to squeeze through pre-existing channels one-tenth the diameter of the Transfersome (Fig. 19.2) allowing them to spontaneously penetrate the stratum corneum. Cevc and Blume suggested that the driving force for skin penetration of these elastic vesicles is the osmotic gradient caused by the difference in water content between the relatively dehydrated skin surface (approximately 10–30 % water) and the highly hydrated viable epidermis (approximately 70 % water) (Fig. 19.1: Kumar et al. 2012; Cevc and Blume 1992; Cevc 1996). A topically applied Transfersome suspension is subject to evaporation on the skin surface; therefore, the Transfersomes penetrate to the deeper skin tissues to avoid dehydration (Cevc et al. 2002). Conventional liposomes remain near the skin surface, dehydrate and fuse with the skin lipids, whilst deformable Transfersomes 'squeeze' through stratum corneum lipid lamellar regions penetrating deeper to follow the osmotic gradient. Consequently, Transfersomes should not be applied under occlusion, as this would decrease the osmotic effect (Cevc et al. 2002; Honeywell-Nguyen et al. 2003). El Maghraby et al. (1999) suggested an alternative or complementary mechanism that occlusion would swell the corneocytes, reducing the size of channels in the stratum corneum thus reducing access to elastic liposomes.

There is still debate as to whether Transfersomes and deformable vesicles act as carrier systems by penetrating intact through the stratum corneum and viable epidermis into the systemic circulation. Evidence based on confocal laser scanning microscopy (CLSM) studies has shown intact elastic vesicles can penetrate the deeper layers of the stratum corneum in some cases (Schatzlein and Cevc 1998; Cevc et al. 2002) but not in others (van den Bergh et al. 1999a, b). Freeze fracture electron microscopy (FFEM) of tape-stripped human skin has also been used to visualise topically applied elastic vesicles (L-595/PEG-8-L/sulfosuccinate or L-595/Tween 20/sulfosuccinate)

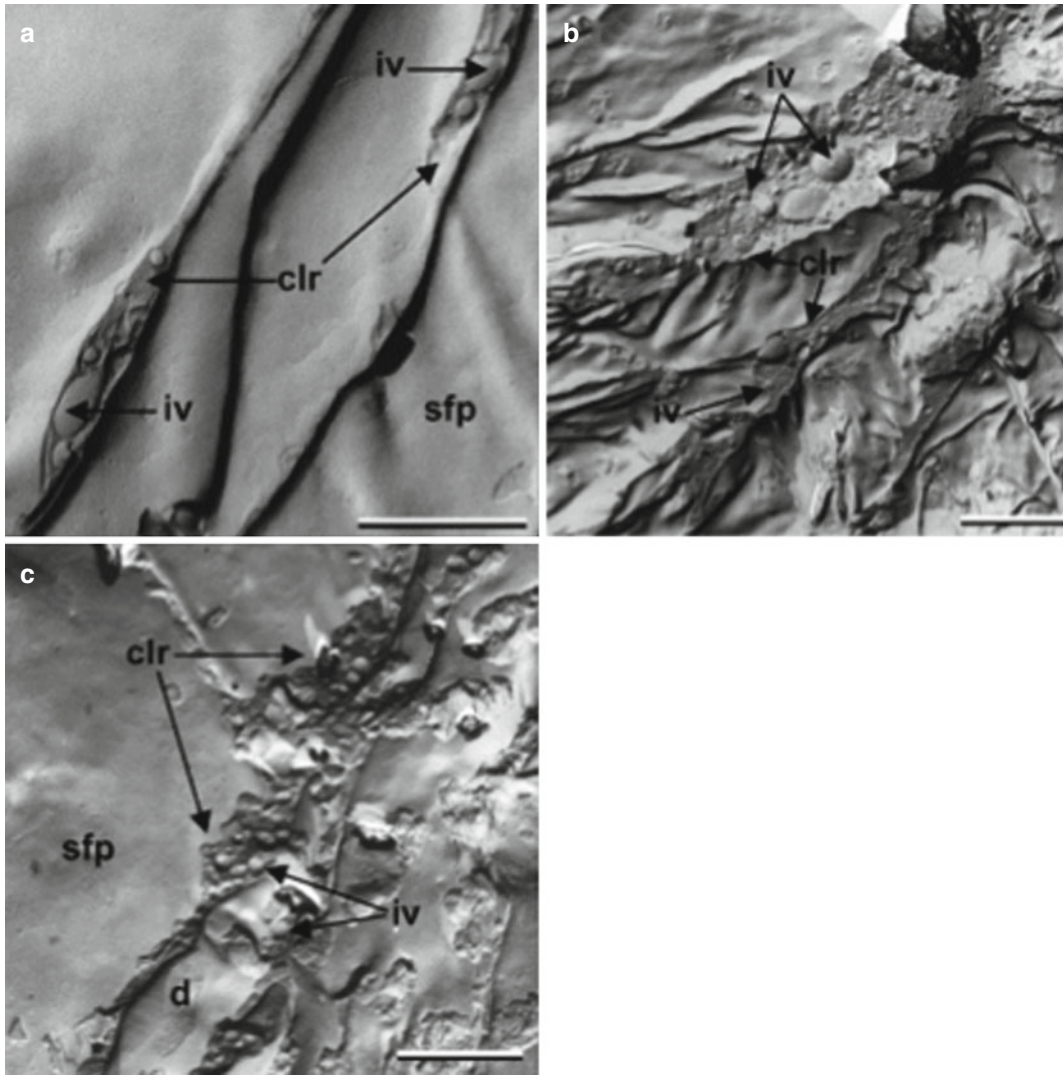

Fig. 19.2 In vivo interactions between elastic vesicles and human skin in the deeper layers of the SC. (**a**) Micrograph of the 9th tape strip of skin treated with L-595/PEG-8-L/sulfosuccinate (50:50:5) elastic vesicles. (**b**, **c**) Micrographs of the 9th tape strip of skin treated with L-595/Tween 20/sulfosuccinate (60:40:5) elastic vesicles. For both elastic vesicle compositions, channel-like regions can be seen containing vesicular structures. Although fused vesicle material is present, intact vesicles were also clearly seen. This suggests that elastic vesicles can enter the deeper layers of the SC within 1 h of vesicle application. *SFP* smooth fracture planes, *D* desmosome, *CLR* channel-like regions, *IV* intact vesicles. Scale bar represents 1 μm (Reproduced from Loan Honeywell-Nguyen et al. (2002) with permission from Elsevier)

up to the ninth and fifteenth tape strip following unoccluded application of the formulation for 1 and 4 h, respectively (Fig. 19.2) (Honeywell-Nguyen et al. 2002, 2003). Throughout these studies, which were designed to investigate the possible interaction between vesicles and the skin, Bouwstra's group found no evidence of intact elastic vesicles beyond the stratum corneum. Consequently, there is considerable scepticism regarding the claims that intact elastic vesicles penetrated through the stratum corneum and the underlying viable epidermis to carry substantial drug payloads into the blood circulation.

The basic principle of Cevc's original Transfersome approach was to incorporate an 'edge activator' in the form of a surfactant that would destabilise the phospholipid bilayers of the vesicle thereby increasing deformability in the stratum corneum. Cevc utilised phosphatidylcholine in combination with the surfactant sodium cholate (Cevc 1996), but many other compositions of elastic vesicles have also been developed and evaluated. For example, van den Bergh et al. introduced a series of elastic and rigid liquid-state vesicles consisting of the bilayer-forming surfactant L-595 (sucrose laurate ester), the micelle forming surfactant PEG-8-L (octaoxyethylene laurate ester) and a charge inducer (sodium bistridecyl sulfosuccinate) (van den Bergh et al. 1999a, b). These 'elastic niosomes' were shown to be effective only when the drug molecules were entrapped within the vesicles, demonstrating that they acted as carriers rather than their components solely acting as penetration enhancers. Trotta et al. formed flexible vesicles with soy phosphatidylcholine or hydrogenated lecithin with dipotassium glycyrrhizinate as the surfactant (Trotta et al. 2002, 2004). Song and Kim used the cationic lipid 1,2-diolyl-3-triethylammonium-propane (DOTAP) in combination with the nonionic surfactant Tween 20 to form positively charged elastic vesicles aimed to be attracted to the negatively charged skin (Song and Kim 2006). They concluded that the positively charged elastic vesicles provided greater skin retention by interaction with the negatively charged skin, a finding that has been previously demonstrated with conventional liposomes (Kirjavainen et al. 1996).

The second generation of elastic liposomes incorporated penetration enhancers such as ethanol (Touitou 1996), propylene glycol (Manconi et al. 2011), terpenes (Dragicevic-Curic et al. 2008), oleic acid (El Maghraby et al. 2004) and Transcutol® (Manconi et al. 2012), within the vesicle structure. El Maghraby et al. (2004) investigated chemical penetration enhancers such as oleic acid and limonene as the edge activators. Using differential scanning calorimetry (DSC), they showed that the phase transition temperature of the lipid membranes was reduced in the presence of surfactants, oleic acid and limonene and increased in the presence of cholesterol. Manconi et al. (2012) demonstrated penetration of intact penetration enhancer vesicles (PEVs) containing Transcutol (10, 20 and 30 %) within the stratum corneum under CLSM, with enhanced skin transport of diclofenac sodium compared to control vesicles. Using small and wide-angle X-ray scattering, they showed an intercalating incorporation of Transcutol molecules into the phospholipid lamellae, improving bilayer fluidity and decreasing the bilayer thickness compared to control vesicles. They concluded that drug transport was enhanced by penetration of intact PEVs through the stratum corneum due to the synergistic effect of the flexible vesicles and the ability of the penetration enhancer to reduce the barrier properties.

Invasomes are PEVs composed of phosphatidylcholine, ethanol and mixtures of terpenes such as limonene, citral and cineole (Dragicevic-Curic et al. 2008). The presence of ethanol and terpenes increases the invasome flexibility as demonstrated using cryo-electron microscopy. The authors have proposed that they act by a synergistic effect of the liposomes, ethanol and terpenes: some of the vesicles fragment during their penetration into the stratum corneum layers to release terpenes and phospholipids, which act as penetration enhancers in combination with the ethanol to fluidise the intercellular lipids. The authors proposed that the combination of a fluidised stratum corneum, the high deformability of the invasomes and the presence of the transepidermal osmotic gradient facilitate the penetration of some small intact invasomes, which were not fragmented during their penetration.

Ethosomes are a class of vesicles based on high ethanol content. Ethanol is known to act as a skin penetration enhancer at concentrations up to approximately 60 % and was first included in liposomes to form ethosomes by Touitou (1996). Enhanced penetration to deep tissues and the systemic circulation has been reported for a range of drugs encapsulated in ethosomes, e.g. minoxidil, testosterone, 5-fluorouracil, ligustrazine, diclofenac and heparin (Touitou et al. 2001; Godin

and Touitou 2003; Ainbinder and Touitou 2005; Elsayed et al. 2006; Song and Kim 2006; Dubey et al. 2007; Mishra et al. 2007; Liu et al. 2011; Puri and Jain 2012). Comparison of ethosomal vesicles with hydroalcoholic formulations has demonstrated that enhanced delivery is achieved with the vesicles, and therefore, the enhancement is not due solely to the presence of ethanol (Touitou et al. 2000). The inclusion of high concentrations of ethanol (typically 20–45 %) in the phosphatidylcholine structure creates bilayers that are flexible and have low melting transition. Touitou suggested that it is this flexibility that allows the soft, malleable ethosomes to deposit within the stratum corneum bilayer lipids, which are then potentially fluidised by the ethanol and phospholipid formulation components, thus promoting penetration into the deeper layers of the skin (Touitou et al. 2000; Elsayed et al. 2006; Dubey et al. 2007). One important difference between ethosomes and other elastic vesicles is that ethosomes have been shown to enhance skin penetration under both non-occlusive (Dayan and Touitou 2000) and occlusive conditions (Dubey et al. 2007). Geusens et al (Geusens et al. 2010) recently reported delivery of siRNA (scrambled siRNA, 5′-AUU-AUC-UAG-GAG-AUA-UCA-3′ and siRNA against MyosinVa exon F, 5′-GUAUCAAUCAUAUCGGAUU-3′) into human skin from ultraflexible 58 nm nanosomes composed of the cationic lipid 1,2-dioleoyl-3-trimethylammonium propane chloride (DOTAP), cholesterol, sodium cholate and 30 % ethanol. They termed their nanosomes SECosomes (surfactant-ethanol-cholesterol-osomes). Multiphoton microscopy with fluorescence lifetime imaging (MPM-FLIM) was used to visualise the distribution of the SECosomes within the skin. They concluded that whilst the exact mechanism of transport into the skin is unclear, the synergistic effect of the surfactant and ethanol contributed to the ultradeformability and permeation into the skin.

Recently Cevc (2012) suggested a third generation of elastic vesicles and a method for their rational design to provide enhanced physicochemical and penetration characteristics. Clearly, this ongoing development of elastic vesicle formulations utilising a wider range of components is likely to increase their application and offer opportunities for peptide and protein delivery to the skin.

19.3.1 Mechanism of Elastic Vesicles Transport in the Skin

Elastic vesicles offer the advantages of the liposome lipid bilayer carrier structure whilst incorporating a surfactant that permits elasticity and deformation of the bilayer. This deformation allows the elastic vesicle to squeeze through small spaces thereby facilitating permeation within the stratum corneum lipid bilayers. Ultrastructural changes in the form of ‘threadlike channels’ have been identified within the stratum corneum due to the presence of elastic vesicles (Fig. 19.1) (Honeywell-Nguyen et al. 2002; Honeywell-Nguyen and Bouwstra 2005). Untreated skin and skin treated with conventional rigid liposomes did not display similar channels. Distinct regions of lamellar stacks derived from vesicles have been observed in the intercellular spaces of the stratum corneum (van den Bergh et al. 1999a, b; Jain et al. 2006). This ability to alter the structure of the stratum corneum is the main mechanism by which elastic vesicles permeate the stratum corneum and therefore their primary mechanism of permeation enhancement. The presence of intact elastic vesicles well within the stratum corneum has been clearly demonstrated, but claims that intact elastic vesicles can permeate through the viable epidermis to the blood circulation are more contentious.

19.3.2 Elastic Vesicles: Transdermal Delivery of Peptides and Proteins

The application of elastic liposomes to peptide and protein delivery has been limited. Some promising findings with insulin and other peptides were reported a decade ago but have not led to further development. In recent years, there has been interest in the application of elastic vesicles for transcutaneous immunisation, in particular coupling elastic liposomes with other permeation enhancement strategies.

Due to the size (molecular weight approximately 6000 Da) and polarity of insulin, passive permeation of a standard formulation across intact human or animal skin is negligible. Cevc et al. reported that 90–110 nm diameter Transfersomes composed of soybean phosphatidylcholine (SPC) incorporating sodium cholate (SC) and ethanol could deliver insulin to the systemic circulation in therapeutic amounts equivalent to a subcutaneous injection (Cevc et al. 1998; Cevc 2003). Preliminary experiments involved in vitro skin permeation and determination of glucose lowering in mice. Radiolabelled insulin delivered in Transfersomes reduced blood glucose levels in mice with a 30 min lag time, but overall efficacy of delivery was comparable to a subcutaneous injection. The optimal formulation consisting of 8.8:1.2 SPC:SC with approximately 10 % ethanol and incorporating human recombinant insulin (termed Transfersulin®) was applied to the intact skin on the inner forearm of human volunteers. The insulin:C-peptide ratio was determined over time by radioimmunoassay of blood samples. Although there were problems with the assay method and large variability in results, the authors did report that there was evidence of blood glucose lowering following Transfersulin administration. Conventional liposomes and mixed micelle formulations did not deliver insulin, demonstrating that the penetration achieved by Transfersomes was not due to the components of the formulation. In a subsequent study (Cevc 2003), Transfersulin was administered to diabetic patients following the withdrawal of their regular insulin. A number of formulations were tested with varied results. The authors reported that in some cases, the Transfersulin formulation did maintain blood glucose in the desired range. No further research on Transfersulin has been published since 2003. Cevc (2003) also reported similar biodistribution profiles of ^{125}I for radiolabelled calcitonin, interferon-γ and serum albumin (molecular weights 3432, 16,879, 66,463 Da, respectively) following Transfersome-mediated skin application and subcutaneous injection to mice. The Transfersome technology is owned by Idea AG, Germany. Recent press releases suggest that the company is focussed on small molecule delivery rather than peptides/proteins.

Guo et al. (2000a) reported successful transdermal insulin delivery with elastic vesicles composed of lecithin (phosphatidylcholine) and sodium cholate. Conventional and elastic liposomes of similar size (74 and 87 nm, respectively) were applied unoccluded to mouse abdominal skin in vivo. Blood glucose decrease following elastic vesicle application was 21.4 ± 10.2 % at 1 h and reached 61.5 ± 9.0 % at 5 h. However, the results were confounded because both flexible vesicle formulations with and without insulin appeared to lower blood glucose. The authors concluded that this was most likely due to contamination. Conventional vesicles, insulin solution and saline provided no hypoglycaemic effect. Guo et al. (2000b) also reported delivery of the peptide Cyclosporin A (1201 Da) from a similar flexible vesicle formulation across mouse skin in vitro and in vivo. Vesicles with and without the incorporation of sodium cholate and sodium cholate micelles were investigated. Only the elastic vesicles transferred Cyclosporin A to the receptor solution and blood circulation in measurable amounts. The authors also reported that hydration of the skin decreased the efficacy of the flexible vesicles in delivering the peptide across the skin.

Transcutaneous immunisation (TCI) is attractive because of the presence of Langerhans cells and dendritic cells in the epidermis and dermis, respectively, which have important roles in the capture, uptake and processing of antigens. Delivery of vaccine to these regions can therefore produce both a mucosal and systemic response, thus providing an advantage over intramuscular injection (IM). Cevc's group first evaluated the potential of elastic vesicles for noninvasive vaccine delivery. Potent humoral immune responses were induced in murine models to antigens such as human serum albumin and gap junction protein applied topically in Transfersomes (Paul et al. 1995, 1998). Gupta et al. (2005a, b) reported that the serum IgG antibody titre generated in response to administration of tetanus toxoid in elastic vesicles, niosomes and conventional liposomes to the shaved skin of rats was compared to an alum-absorbed tetanus toxoid given intramuscularly. Two applications of tetanus toxoid in elastic vesicles given 28 days apart elicited an

immune response that was equivalent to that produced by the intramuscular injection. Niosome and conventional liposome formulations elicited weaker immune responses. When measured across nude rat skin in vitro over a 48 h period, the cumulative permeation of tetanus toxoid was approximately 16.4, 12.5 and 10.7 % of applied dose for the elastic vesicle, niosome and liposome formulations, respectively.

The same group (Mishra et al. 2006) also reported successful delivery of proteinaceous antigens in elastic vesicles for transcutaneous vaccination. Ex vivo cellular uptake and fluorescence microscopy studies demonstrated skin permeation, biodistribution and efficient delivery of antigens to the immunocompetent Langerhans cells and lymphatics. The immune response elicited by topically applied hepatitis B surface antigen (HBsAg)-loaded elastic liposomes (composed of phosphatidylcholine, Span 80 and ethanol) was compared to IM administered alum-adsorbed HBsAg solution, topically applied plain HBsAg solution and a physical mixture of HBsAg and elastic liposomes in mice. Elastic liposomes induced a comparable systemic response to the IM injection as demonstrated by IgG titres. However, much higher secretory IgA titres against HBsAg were obtained with the elastic liposomal formulation. Bal et al. have drawn attention to the experimental protocol in this study, pointing out that as no washing step was included after topical antigen application on the back of the mice, oral delivery following grooming by the rodents may have contributed (Bal et al. 2010). Their group reported that a 1 h topical application of cationic elastic liposomes (composed of phosphatidylcholine, Span 80 and 1,2-dioleoyl-3-trimethylammonium-propane chloride salt [DOTAP]) loaded with diphtheria toxin to anaesthetised mice, did not improve the immune response.

19.4 Combination of Vesicles and Microneedles

The combination of microneedle pretreatment or cotreatment of the skin to reduce the stratum corneum barrier, followed by topical application of a vesicle formulation, has been evaluated for vaccine delivery. Microneedles create channels in the stratum corneum through which topically applied molecules can flow to more readily access the epidermis. Ding et al. showed a significant improvement in immune response to diphtheria toxoid applied as elastic vesicles associated to BALB/c mice following pretreatment of the skin with a microneedle array, using cholera toxoid as an adjuvant (Ding et al. 2011). They investigated a range of vesicle compositions including cationic and anionic vesicles of 150 and 100 nm, respectively. Vesicle compositions applied to both intact and microneedle-treated skin did not enhance immunogenicity. They concluded that microneedle pretreatment and cholera toxin, but not antigen association to vesicles, enhanced the immunogenicity of topically applied diphtheria toxoid. In a recent study by Hirschberg et al. (2012), vesicle association did prove favourable for enhanced immunogenicity. HBsAg was incorporated into two vesicle formulations composed of L-595/PEG-8-L (75 nm) and phosphatidylcholine/Span 80 (170 nm). The vesicles induced an antibody response in mice only when applied to microneedle-pretreated skin. Despite the larger size, the phosphatidylcholine vesicles induced the highest immune response, which was related to a much higher association/entrapment of HBsAg in comparison to the surfactant vesicles (29 % and 79 % HBsAg association, respectively). As with the previous study, the response was increased by the cholera toxoid adjuvant. Guo et al. (2013) developed a dissolving polyvinylpyrrolidone microneedle array, with the microneedle tips loaded with antigen and adjuvant encapsulated in liposomes that dissolve within 3 min in the skin. Mice immunised transcutaneously with the array containing ovalbumin (OVA) as a model antigen and CpG oligodeoxynucleotides as adjuvant in cationic liposomes (composed of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine, cholesterol and dimethyldioctadecylammonium) showed significantly higher anti-OVA IgG antibody level than conventional intramuscular injection with OVA solution. These studies suggest that TCI by microneedles holds promise with presentation in liposomal formulation potentially enhancing the immune response.

Conclusion

There is evidence that elastic vesicles can enhance delivery of small molecules into and through the skin. They also hold potential for the delivery of peptides into the skin for the dermatological and cosmeceutical applications. However, the evidence for systemic delivery of therapeutically relevant quantities of proteins, such as insulin and vaccine antigens, is not strong. Despite some promising early reports, these have not been substantiated in more recent, well-conducted studies. There is the potential to combine vesicle formulation with microneedle pretreatment, but there is not clear evidence that the vesicle formulation offers a clear advantage over a well-formulated solution. A number of Transfersome products are now in advanced clinical trials or marketing phase, such as Diractin® (Idea AG, Munich) for enhanced delivery of ketoprofen in the management of osteoarthritis. The deformable vesicle concept can be applied to a variety of compositions with the potential to optimise the skin deposition and permeability of a range of therapeutic molecules. There is continuing interest in elastic vesicle delivery with development of optimised vesicle components that offer advantages with respect to improved delivery and reduced irritation profile. Indeed, there is considerable scope for exploring innovative compositions that incorporate suitable chemical penetration enhancers to offer synergistic effects within the stratum corneum. It is likely that a number of elastic vesicle-based products incorporating both small molecules and peptides for dermal applications will be developed in the future.

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20 Permeation Enhancement by Molecular Organization Switching (MOS): Biphasic Vesicles for the Cutaneous Delivery of Proteins

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20.1 Introduction

Basic research and technology development into noninvasive drug delivery has intensified in recent years. There are various pharmaceutical approaches suitable for topical delivery (Fig. 20.1). The choice is dependent on the properties of the drug, target site of delivery, and whether there is a requirement for pharmacokinetic modulation of the absorption rate. Drug release from a matrix on the skin surface provides a metering mechanism for drugs that can permeate the skin due to their advantageous physicochemical properties such as suitable lipophilicity (oil/water partition coefficient of 10–1000), molecular weight <500 Da, melting point <200 °C, pH of the saturated aqueous solution of 5–9, and a low deliverable dose of <10 mg/day (Naik et al. 2000). Encapsulation into particulate delivery systems provides a way to concentrate the drug close to the skin surface thereby increasing the concentration gradient and drug absorption. The use of penetration/permeation enhancers is necessary in most cases to achieve pharmacological levels of drug delivery from any matrix or formulation. Additionally, properly designed delivery systems (e.g., liposomes, nanoemulsions, lipidic or polymeric nanoparticles) that interact with the skin and themselves act as carriers, penetration/permeation enhancers, drug release controllers, and targeting agents are the most efficient percutaneous dosage forms (Fig. 20.1).

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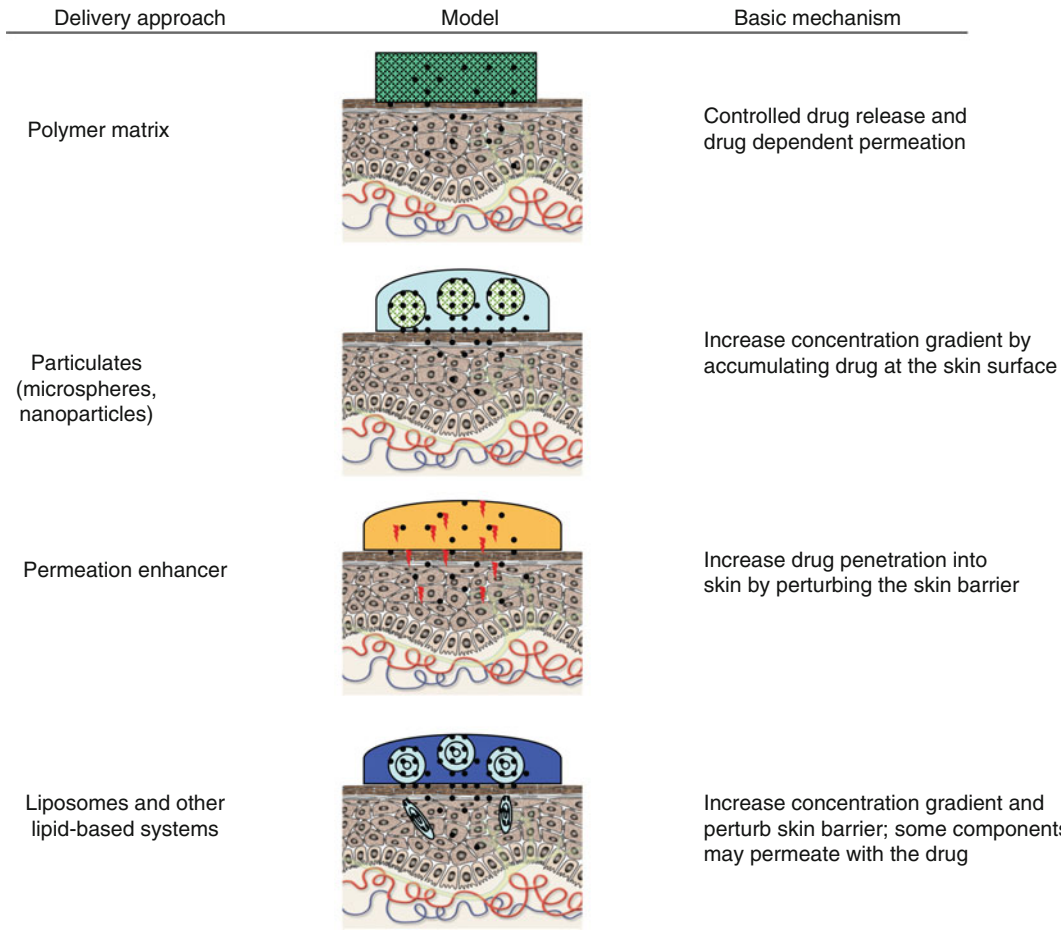

Fig. 20.1 Approaches and mechanisms of drug delivery into the skin. The physicochemical properties (e.g., solubility) of the drug to be delivered influence the choice of delivery approach and formulation which is used and the mechanism of delivery into and diffusion within the skin

Delivery into and through the skin represents an important method by which biologics, such as peptides, recombinant proteins, antibodies, and nucleic acids, could be administered for the treatment of dermatological conditions, cutaneous gene therapy, and potentially for the systemic delivery of these macromolecules. Sustained dermal and transdermal delivery of macromolecules would have many advantages compared with administration by injection and other short-acting conventional dosage forms, but the low permeability of compounds with molecular weights greater than about 500–1000 Da into or through the skin, combined with a lack of efficient and safe methods, limits this route of administration.

The development of pharmaceutical products containing biological molecules for topical drug delivery provides additional challenges. Many macromolecules, because of their inherent instability of storage, incompatibility with many inactive ingredients routinely used in pharmaceutical industry, and limited availability of stability literature, lead to an increase in time and cost to develop commercially viable products with a sufficient shelf life. Unlike small molecules, pharmaceutical products containing biologicals must also be evaluated for immunological responses.

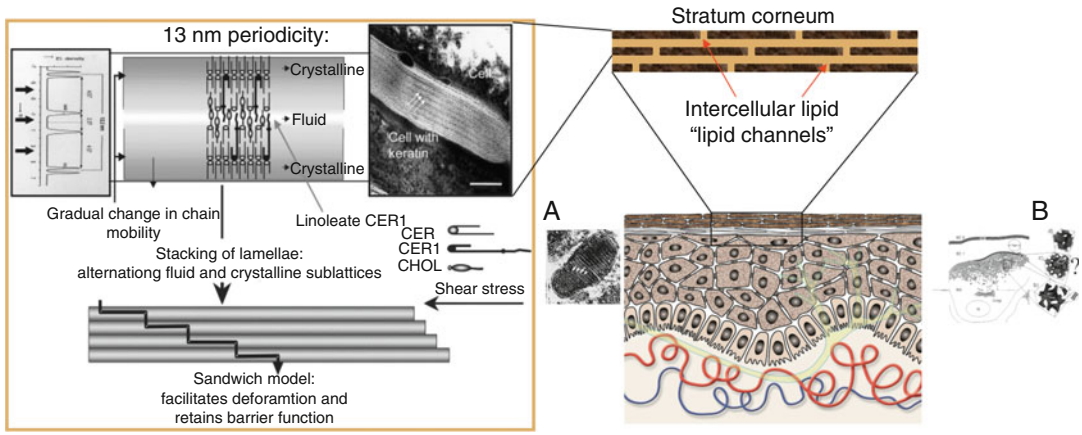

Fig. 20.2 Lamellar structure of the intercellular lipid channels (Reproduced with permission from Bouwstra et al. (2003)). Hypothetical lamellar bodies (Landmann 1988) (*A*) and cubic lipid structures (*B*) within the stratum granulosum as precursors of intercellular lipid channels (Norlen 2001a, b)

20.2 Drug Delivery and Structural Aspects of Skin Barrier

Three basic approaches are used to facilitate absorption of molecules into the skin: physical methods, chemical penetration enhancers, and/or delivery systems. Selection of a suitable chemical enhancer for a drug is based mostly on *in vitro* or *in vivo* screening studies that quantitatively measure the extent of skin penetration enhancement. More than 300 chemical enhancers are known, which are classified into groups based on chemical structure or their mechanism of permeation enhancement (Williams and Barry 1992; Chattaraj and Walker 1995).

The mechanisms of chemical permeation have been the subject of extensive study for over a century, but even deeper understanding is necessary. The skin's three main layers, the epidermis, dermis, and subcutaneous tissue, each play a role in limiting drug delivery. The uppermost layer of the epidermis, the stratum corneum (a nonviable, 10–15 μm thick layer), is the main limiting barrier to drug penetration. The stratum corneum, represented by a "brick and mortar" model (Elias 1983, 1990; Tojo 1987; Abraham and Downing 1990), consists of corneocytes (bricks) and intercellular spaces filled with lipids (mortar), the former being a hydrophilic domain and the latter hydrophobic (Kuntsche et al. 2008). Absorption may occur through: (1) the intracellular route, which is more typical of hydrophilic substances; (2) the intercellular route, which is common for lipophilic substances; or (3) the appendages (hair follicles, sweat ducts). The main barrier function of the stratum corneum is due to the compact stacking of corneocytes and the multilamellar and hydrophobic nature of the intercellular lipids. In humans, the intercellular lipids, which make up 8–10 % of the total stratum corneum weight (Schurer et al. 1991), consist of fatty acids (mainly C22 and 24, 15–25 %), long-chain ceramides (CER1-CER8) (mostly C24-26, 35–50 %, and CER1 and CER4 with C30-32), cholesterol (15–25 %), and cholesterol sulfate (5–10 %) (Lampe et al. 1983; Lisziewicz et al. 2007; Bouwstra et al. 2001) .

The formation and molecular organization of the stratum corneum lipids can be described by filling of the intercellular spaces with the extruded lamellar bodies (Landmann 1986) or lipids produced in cubic phase (Norlen 2001a) from cells of the stratum granulosum (Fig. 20.2). The *Landmann model* (Landmann 1986) suggested that lamellar granules produced by granular cells in the upper viable epidermis fuse with the cell

membranes and expelled into the extracellular spaces to form uninterrupted sheets in a bilayer configuration. This was confirmed by Swartzendruber et al. (1989) who showed that the pattern of lamellar bodies (stacks of flattened liposomes) located inside these granules can be recognized in the intercellular spaces. The bilayer structure of intercellular lipids is based on the Landmann unit of two stacked lipid bilayers composed of different ceramide molecules (CER1-CER8) in an interdigitated arrangement with a periodicity of approximately 13 nm (Robson et al. 1994; Bouwstra et al. 2003; Swartzendruber et al. 1989). More recent studies indicate that the structure of lipids that are being extruded during barrier lipid formation may be in other polymorphic states such as cubic phase which is a bicontinuous structure where amphipatic molecules are arranged in a porous connected bilayer structure in three dimensions (Norlen 2001a). Norlen's *membrane folding model* proposed that intercellular lipid channel formation via cubic-lamellar phase transition is more energetically favorable (Norlen 2001a, b) than lamellar granule fusion.

20.3 Skin Permeation Enhancement: Structural Theory

In general, absorption enhancement through lipid channels depends on the ability of the enhancer to integrate with the existing lipids and create a perturbed microenvironment based on various possible mechanisms (Hadgraft et al. 1992; Hadgraft 1996; Marjukka Suhonen et al. 1999) such as (1) alteration of lipid phase fluidity, (2) enhancement of solubility characteristics of the skin for the drug to be delivered, (3) creation of a disordering effect among the alkyl chains of skin lipids, and (4) localized separation of lipid domains to create hydrophilic pores. Currently known penetration enhancers appear to induce a penetration pathway within the stratum corneum that is the result of the disorganization of intercellular lipids. This may be caused by extraction of lipids by solvents (Kaushik et al. 2010; Foldvari 2000) or surfactants (Foldvari 2000) and integration of the permeation enhancer into the lipid channels, e.g., in the case of terpenes, surfactants, 1-dodecylazacycloheptan-2-one (Azone®) and derivatives, soft enhancers of percutaneous absorption (SEPA), liposomes, and other vesicular delivery systems (Ibrahim and Li 2009, 2010; Yerramsetty et al. 2010; Karande et al. 2004, 2005, 2007).

20.4 Biphasic Vesicles

Biphasic vesicles were originally designed as a multicompartmental lipid vesicle system for enhanced encapsulation of drugs. However, as a pharmaceutical multifunctional delivery system, it also has flexible components that allow for the development of a fully tailored topically applied dosage form. The permanent components include the submicron emulsion system and the phospholipid bilayers. In addition, the flexible components include viscosity-increasing agents and emollients, which can be incorporated into the submicron emulsion systems and permeation enhancers, which can be added to the emulsion system or the bilayer forming lipids. The stability of the biphasic vesicles was improved by increasing their mechanical strength while adding cholesterol. The resulting vesicles can be considered a structurally organized form of synergistic mixture of permeation enhancers (Fig. 20.3).

The importance of being structurally organized, as opposed to just simply a mixture of the same materials, is supported by data indicating the diminished delivery capacity of the simple mixture of components. Recent studies on the interaction of biphasic vesicles with stratum corneum showed lipid organizational changes within the stratum corneum, which was associated with increased delivery of interferon alpha (IFNα), a 19 kDa protein (Foldvari et al. 2010, 2011). Using the biphasic vesicle technology, a topical dosage form of IFNα2b, a potent cytokine that possesses antiviral, immunomodulating, and antiproliferative activities with activity against human papilloma virus, has been developed and is being studied in humans for the treatment of precancerous lesions (CIN1 and CIN2) caused

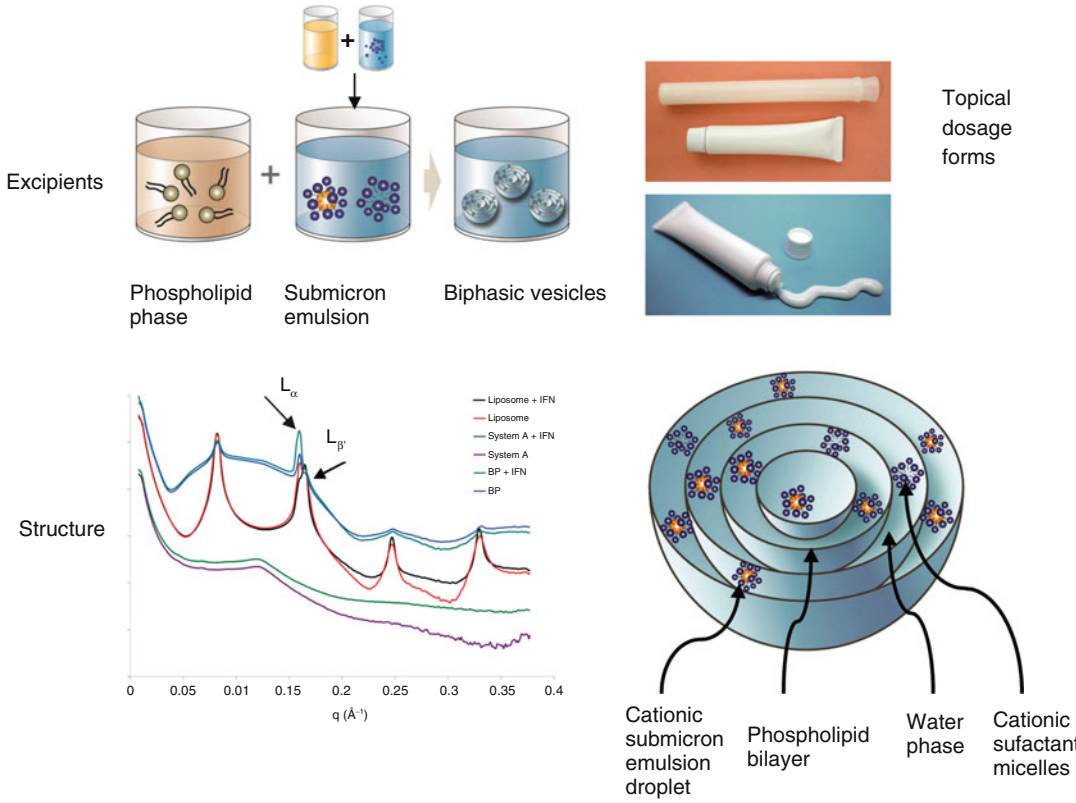

Fig. 20.3 Biphasic vesicle assembly and structure. The formulation process consists of two main steps: the submicron emulsion (made by high-shear homogenization – step 1) is mixed with the phospholipid phase at low-shear conditions (step 2). Topical dosage forms, for skin or intravaginal administration, with different viscosities, can be formulated by adjusting the type and concentration of excipients used. Synchrotron X-ray (SAXS) structural studies indicated that biphasic vesicle structural features contain the reflection patterns of both liposomes and emulsion droplets; however, the bilayer-emulsion droplet spacings are characteristic of entrapped objects (as depicted in the biphasic vesicle drawing) rather than just a physical mixture of liposomes and emulsion (Modified from Foldvari et al. (2011))

human papillomavirus (HPV) infections in humans (Foldvari and Kumar 2012). The rationale for biphasic vesicle construction stems from the selection of synergistic permeation enhancer combinations and pharmaceutical dosage form development (Fig. 20.4).

Figures 20.5 and 20.6 illustrate the physicochemical and structural changes that occur during biphasic vesicle treatment. The combination of differential scanning calorimetry (DSC), confocal microscopy, and structural SAXS and WAXS measurements suggested that lipid reorganization occurs after biphasic vesicle dosing. DSC studies confirmed the changes in thermotropic behavior of SC lipids after topical treatment with biphasic vesicles. The typically occurring T1, T2, and T3 thermotropic transitions of SC lipids [at 38–42 °C (lipid packing), 69–77 °C (reversible lipid transition), and 78–92 °C (lipids bound to protein), respectively] decreased, whereas Tx (at about 55 °C) prominently increased (Fig. 20.5). An increase in the Tx peak reflects changes in the structural arrangement of the covalently bound lipids around the corneocytes (Gay et al. 1994; Cornwell et al. 1996). A decrease in the T1 peak at 38–42 °C indicates a lipid disordering effect, which may be due to changes to the originally orthorhombic packing of lipids (Foldvari et al. 2010).

In normal SC, the bilayer stacks of intercellular lipids have a regular repeat pattern with a periodicity of about 13 nm and the lipid head-

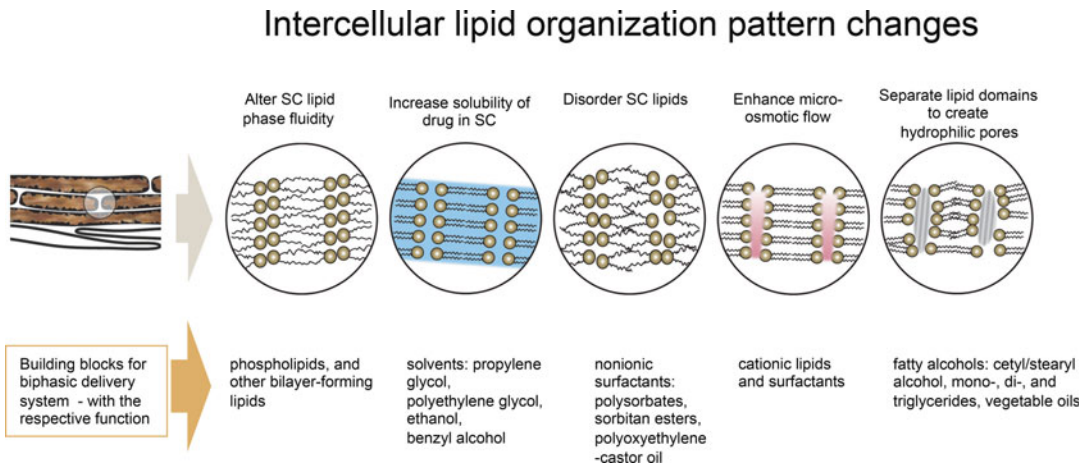

Fig. 20.4 Rationale for the selection of building blocks for biphasic vesicles. A pharmaceutical formulation approach was used to create lipid vesicles, where the main building blocks were selected from ingredients known to enhance transdermal delivery to some degree by acting via different permeation-enhancing mechanisms. Although these ingredients alone are not efficient enough to deliver macromolecules, in the multicompartmental biphasic vesicles, successful dermal and transdermal delivery was achieved because of a structurally and chemically synergistic mechanism

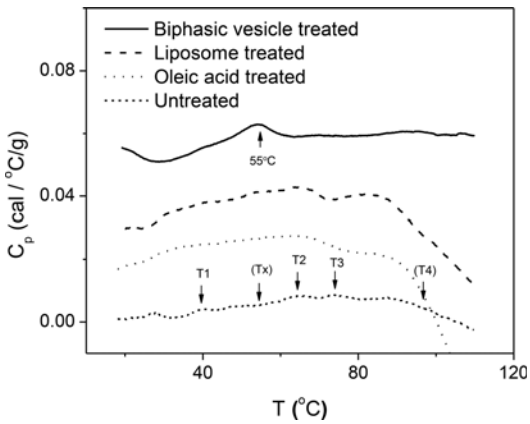

Fig. 20.5 DSC thermograms of SC samples treated with biphasic vesicles and relevant controls (liposomes and oleic acid) (Reproduced with permission from Foldvari et al. (2010))

group packing is in an orthorhombic configuration providing a strong barrier to permeation of substances (Bouwstra et al. 2003; Pilgram et al. 2000). After treatment with liposomes or biphasic vesicle formulations, reflections corresponding to orthorhombic (3.7, 4.1, 4.1 Å), hexagonal (4.1, 4.1, 4.1 Å), and liquid (4.6, 4.6, 4.1 Å) packing could be detected. However, the ratio of the intensities of the peaks at 4.1 and 3.7 Å increased upon treatment with biphasic vesicle formulations, from ~4.3 to 4.9 Å, increasing the probability of a hexagonal packing arrangement contributing to the peak at 4.1 Å (Fig. 20.6). As such, the increase in the ratio of intensities may indicate that the biphasic formulation increased the portion of lipid molecules arranged in a lateral hexagonal packing, corresponding to a reduced packing density of lipids and an increase in permeability (Foldvari et al. 2010).

Foldvari et al. (2010) showed, using SAXS, that after biphasic vesicle treatment, SC lipids show reorganization and enlarged lipid spacings, which correspond to the appearance of a new polymorphic phase within the SC (Fig. 20.7). A Miller plot analysis of the position of the reflections as a function of the Miller indices ($\sqrt{2}$, $\sqrt{3}$, $\sqrt{4}$, $\sqrt{6}$, $\sqrt{8}$, $\sqrt{9}$, and $\sqrt{10}$ reflections) indicated the presence of a *Pn*3*m* bicontinuous cubic phase. The cubic phase is a three-dimensional network of aqueous channels within a lipid matrix on an infinite periodic minimal surface (Fig. 20.8, also see https://secure.msri.org/about/sgp/jim/papers/morphbysymmetry/table/index.html). The aqueous channels of the cubic phase could be conduits for hydrophilic molecules and appear to be compatible with accommodating

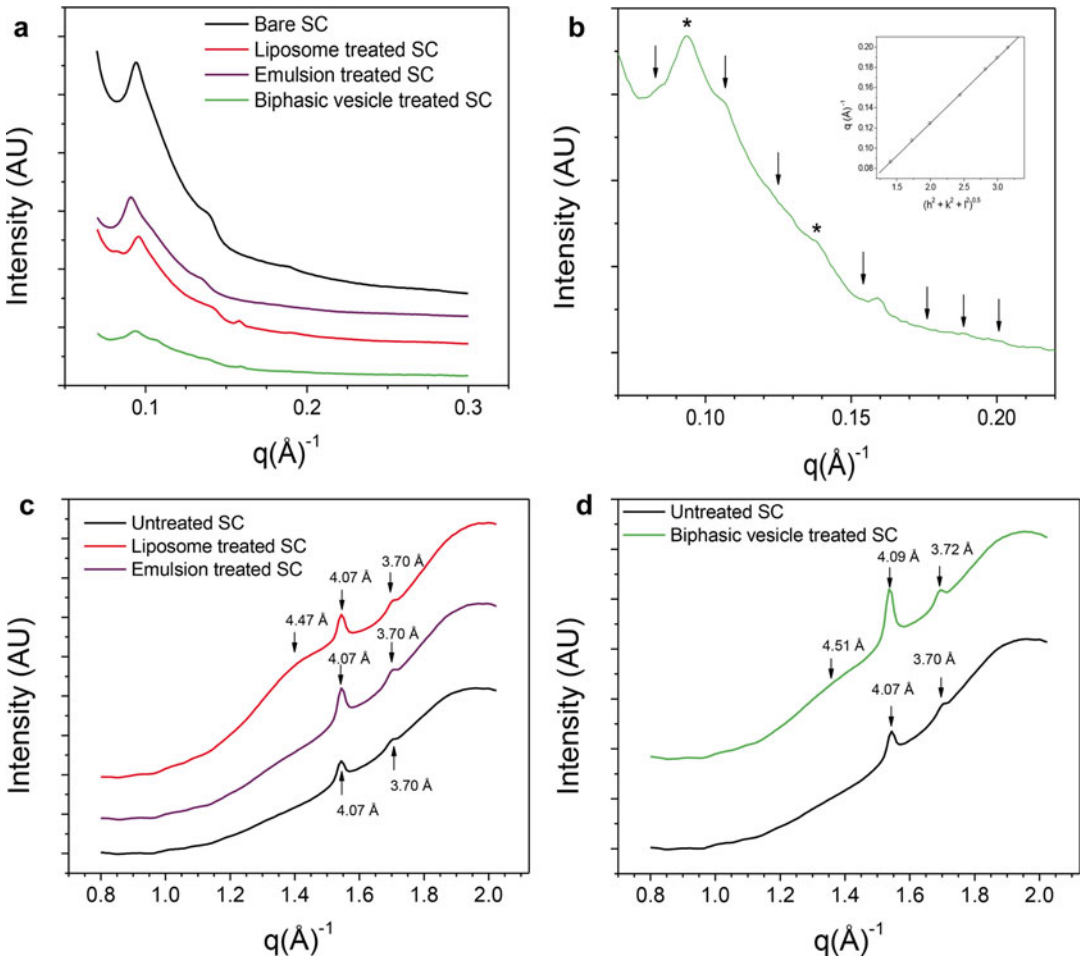

Fig. 20.6 SAXS and WAXS patterns showing the effect of biphasic vesicles and their components (submicrometer emulsion and liposomes) on the lipid organization structure of human SC. The intensity is plotted as a function of q (Å^{-1}), the scattering vector, where q is defined as $4\pi \sin \theta/\lambda$; θ is the scattering angle and λ is the wavelength of the X-rays. SAXS curves of untreated SC and SC treated with biphasic vesicles, liposomes and submicrometer emulsion (**a**); and a scaled graph with an insert of the Miller plot for SC treated with biphasic vesicles showing the reflections ($\sqrt{2}$, $\sqrt{3}$, $\sqrt{4}$, $\sqrt{6}$, $\sqrt{8}$, $\sqrt{9}$, and $\sqrt{10}$) representative of QD cubic phase 224, *Pn3m* (**b**). The reflection observed at 0.158 Å^{-1} is due to crystalline monolauroyllysine, a suspended excipient in the formulation. WAXS curves of untreated SC and SC treated with liposomes and submicrometer emulsion (**c**), and SC treated with biphasic vesicles (**d**) (Reproduced with permission from Foldvari et al. (2010))

various proteins and enzymes (Belrhali et al. 1999; Lunde 2006). Most literature data that describes intercellular permeation of compounds attribute the enhanced permeation to the disordering, disturbance, fluidization, or extraction of lipids in the stratum corneum. However, this novel nanopathway represents a different polymorphic state of the lipids (Foldvari et al. 2010).

The permeation model emerged from the results illustrated in Fig. 20.7.

Biphasic vesicles create an initial disorder among the intercellular lipids, especially among the lipids most closely surrounding the corneocytes (DSC data). In addition, the originally orthorhombic lipid packing within the SC is also affected (WAXS data), indicating appearance of a hexagonal and a liquid state; these latter two are associated with increased permeability. Biphasic vesicles possibly enlarge the lipid spacings through which vesicles/vesicle fragments

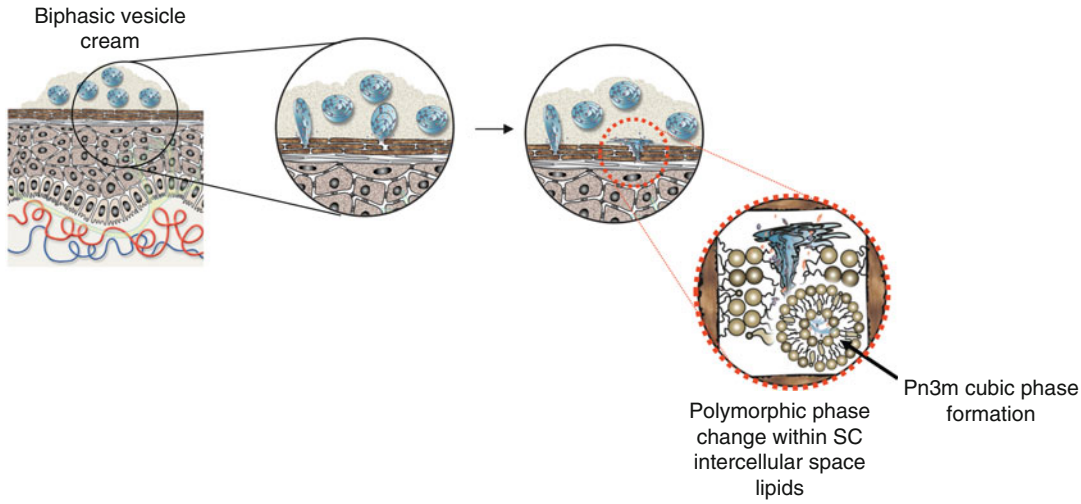

Fig. 20.7 Biphasic vesicle interaction with stratum corneum lipids after application onto the skin in a cream formulation (typically under an occlusion) (Modified from Foldvari et al. (2010))

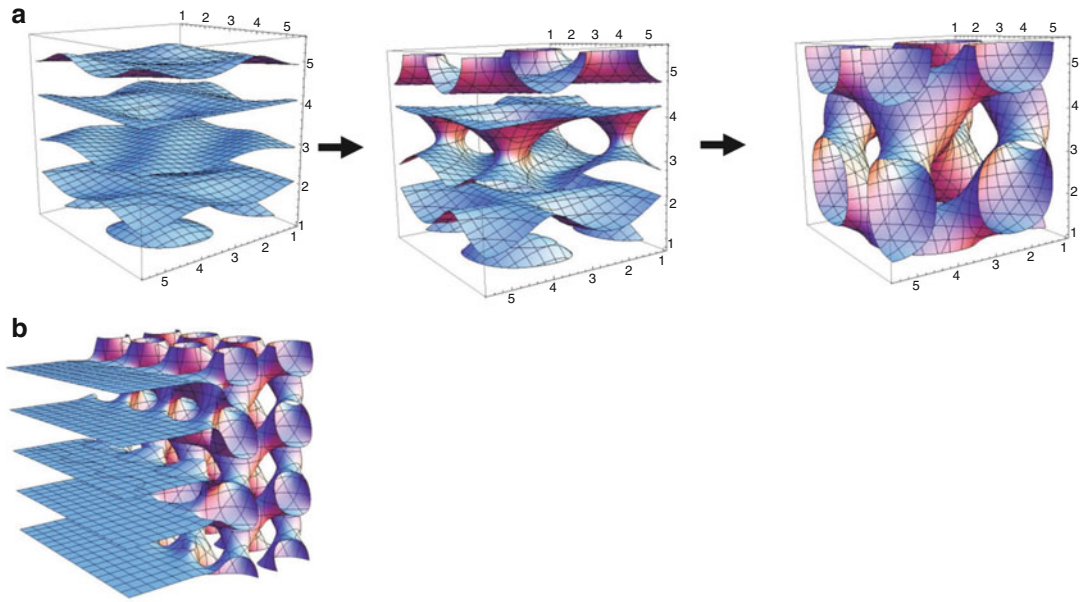

Fig. 20.8 Mathematical models of hypothetical lamellar-to-cubic phase transformation within the SC induced by an external formulation. (**a**) SC lamellar sheets fuse to form three-dimensional Pn3m cubic phase; or (**b**) the biphasic vesicle components merge with the lamellar SC sheets to form Pn3m cubic phase structures (Reproduced with permission from Foldvari et al. (2010))

and encapsulated drug are delivered into the skin and induce a polymorphic change – a molecular organization switching (MOS) – within the SC (supported by SAXS data) evidenced by the appearance of the *Pn3m* bicontinuous cubic phase.

Mathematical modeling of the bilayer-to-cubic phase transformation that may take place upon biphasic vesicle application to the SC is shown in Fig. 20.8. The formation of cubic phase configuration could occur in two ways: (1) by regional fusion of the lamellar lipid layers in the

SC, resulting in a series of molecular reorganization steps and reforming as a three-dimensional cubic phase with aqueous channels (induction model); or (2) by the merging of lamellar phases between the biphasic vesicle lamellar structures and SC lamellar structures to form a cubic phase (merging model) (Foldvari et al. 2010).

20.5 Perspectives on Clinical Application of Topical and Transdermal Protein Delivery Systems

So far, very few topically applied dermal or transdermal delivery systems are available commercially. Examples include Regranex® (becaplermin, recombinant human platelet-derived growth factor, 25 kDa) for lower extremity diabetic ulcers (OMJ Pharmaceuticals/Ortho-McNeil) and topical solution of human thrombin; Recothrom® (ZymoGenetics) and Evithrom® (Omrix/J&J) used to aid hemostasis; and a topical solution of fibrin sealant (Evicel®, Omrix/J&J) used as an adjunct to hemostasis. While these topical protein formulations are applied directly to the site of action or broken skin, their significance in terms of dosage form development and localization of proteins within the skin is important to recognize. The currently advanced level of development is represented by drug device combinations for protein drug delivery. For example, transdermal basal insulin for type I diabetes, parathyroid hormone 1–84 and 1–34, hepatitis B protein antigen vaccine, and IFNα2b utilize the PassPort™ delivery system, a drug/device combination of microporation by thermal ablation and a drug-containing patch by Altea Therapeutics/Nitto Denko, Japan. Other technologies to achieve microporation of the skin include radiofrequency electrical cell ablation (ViaDor™), laser (Precise Laser EpidermAl SystEm; P.L.E.A.S.E.), ultrasound (SonoPrep and a new potentially superior dual-frequency ultrasound technology), and ballistic methods (PowderJect®). Microneedle-based technologies are being developed by several companies, such as solid microstructured transdermal system and hollow microstructured transdermal system (3 M), Macroflux® (Zosano), MicroCor™ (Corium), soluble microneedles (Elegraphy), BD Soluvia™ prefillable microinjection system (Becton Dickinson), and Dermaroller®, a microneedling roller device used mostly for cosmetic applications (DermaRoller).

Conclusion

It is anticipated that the next generation of dermal/transdermal systems for macromolecules may not have to rely on devices but could use synergistic permeation enhancers potentially in patch or patch-free applications for skin and mucosal therapies.

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Vesicular Carriers for Transcutaneous Immunization

21

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Contents

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21.1 Introduction

Immunization or vaccination is an approach of providing defensive obstruction to the body, which helps the body to fight against incoming pathogenic attack/invasion, which the body is immunized against. Thus, immunization creates the ability in the body to fight against various microorganisms and their products (Singh et al. 2002). The role of the skin as a barrier to the external environment depends on the dynamic role of the skin-associated immune-responsive cells, which have been explored and exploited for their active participation in intradermal vaccination (Streilin 1985). Being immunologically rich, the skin offers an attractive route for vaccination (Chen et al. 2001). Vaccination seems to be the most promising strategy for the prevention of many diseases as it is able to promote protective immune response both systemically and at mucosal surfaces. Dermal and transdermal delivery of proteinaceous bioactives encounter massive challenges (Foldvari et al. 1999). Efforts are continuously being done to develop an efficient technique which could deliver peptides and proteins through the skin into the dermal layers. Dermal delivery is having advantages over other routes of administration of peptides, proteins, and antigens as it has the possibility to bypass gastrointestinal degradation and hepatic first-pass elimination, and it shows better patient compliance (Chien 1987).

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21.2 Need for Safer Delivery of Vaccines

Despite the positive effects of vaccination on health, some of the adverse effects associated with injections of vaccines have been realized. Parenteral route causes a significant pain, trauma of needle injection, distress, common adverse reactions, tissue reactions, and an alarming rise of infections, which result in lack of patient compliance. Moreover, administration of vaccines by injections requires syringes, needles, and trained personnel (Pashine 2005). Other delivery routes like nasal (Slutter et al. 2008), percutaneous (Mikszta and Laurent 2008), oral (Simerska et al. 2009), and pulmonary (Giudice and Campbell 2006) route have also been explored for the vaccine administration. Among the different routes, the percutaneous route (vaccination through intact or pretreated skin) is predominantly interesting, as successful and effective immune response can be induced via the skin. In addition, prevention of the direct contact of potent (even slightly toxic) adjuvants with the blood circulation makes the skin a safer route for immune stimulation (Ponvert and Scheinmann 2003). However, the barrier nature of the stratum corneum (uppermost layer of the skin) imposes a major hindrance for the transport of antigens across the skin.

21.3 Transcutaneous Immunization

Transcutaneous immunization (TCI) is a new method for the introduction of antigens into the skin by topical application of vaccine formulations onto the skin, which provides access of antigens to the skin-associated immune system without the use of needles. TCI is an alternative novel method for the conventional vaccination routes, which has been shown to elicit systemic and mucosal antibody responses resulting in the induction of protective immunity against infectious pathogens (Mikszta and Laurent 2008). This is one of the most noteworthy benefits of TCI over the traditional vaccine administration.

It relies on the application of antigen with adjuvant onto the outer layer of the skin and subsequent delivery to underlying densely distributed and potent antigen-presenting cells (APCs), Langerhans cells (LCs), to generate robust

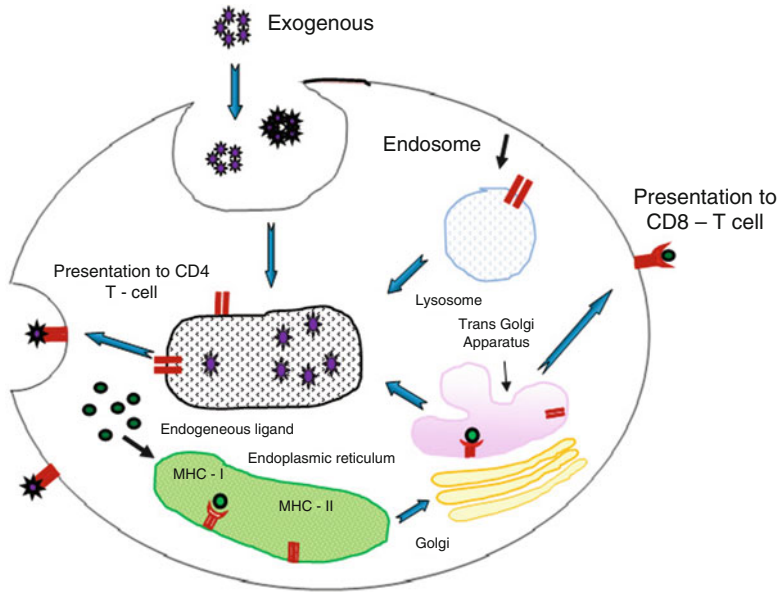

Fig. 21.1 Schematic presentation of the intracellular routes of antigen

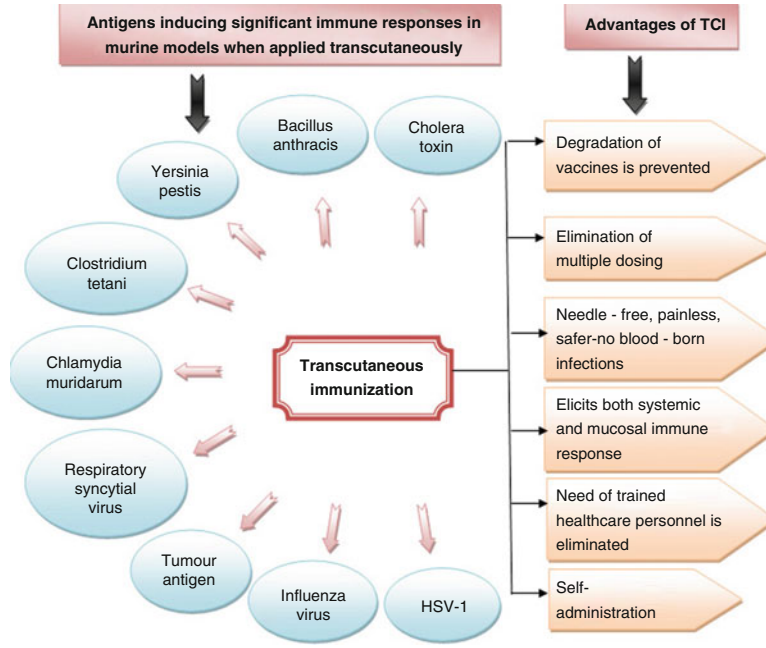

Fig. 21.2 Schematic representation of advantages of TCI and some of the antigens that can induce immune response when applied transcutaneously

immune responses (Fig. 21.1) (Glenn et al. 1999). However, although the vaccination through the skin is an eminent option because of the presence of a high amount of antigen-presenting cells, the stratum corneum hinders vaccine diffusion into the skin. The first investigation of using the skin as a site of vaccine administration was reported in 1997 (Tang et al. 1997), and since then, extensive reports have been published which supported that TCI is an effective vaccine delivery method in animals and humans. Immunocompetent LCs are found in close proximity to the stratum corneum and in abundance along the transdermal penetration pathways, whereas dendritic cells are present in high densities in the dermis (Gupta et al. 2004, 2005).

Presence of these cells makes the skin highly attractive as a target for vaccines by chemical, mechanical, or nanotechnological means and devices thereby promoting access of vaccine antigens to these APCs. Disruption of the stratum corneum using either physical disruption (e.g., tape stripping), penetration enhancers, or cytokine conditioning of the immunization site may provoke immune responses by TCI (Palamara et al. 2004). The advantages of TCI and antigens used for TCI are shown in Fig. 21.2.

21.4 Immunology of the Skin

The skin is an attractive target site for gene therapy protocols, drug, and vaccine delivery. The skin harbors a wide variety of immune cells and elicits a strong immunological response, when it comes in contact with any immunogen. Topical immunization may be an attractive option for both prophylactic and therapeutic vaccines. The skin is an active immune surveillance site rich in potent antigen-presenting dendritic cells (DCs), such as LCs in the epidermis and the immature dendritic cells in the dermis. Other cells present are mast cells in the dermis, resident antigen-presenting cells, and transient inflammatory lymphocytes. These cells altogether function in association with lymph nodes and are responsible for generation of both cellular and humoral immune responses (Gupta et al. 2004).

The skin is well set with a complex network of immune cells, described as "skin-associated lymphoid tissue (SALT)" and "skin immune system," and it constitutes a primary immunological barrier to the external environment. The presence of cytokines which have the capacity to regulate the immune responses confirms the existence of SALT in the skin. The skin immune system is

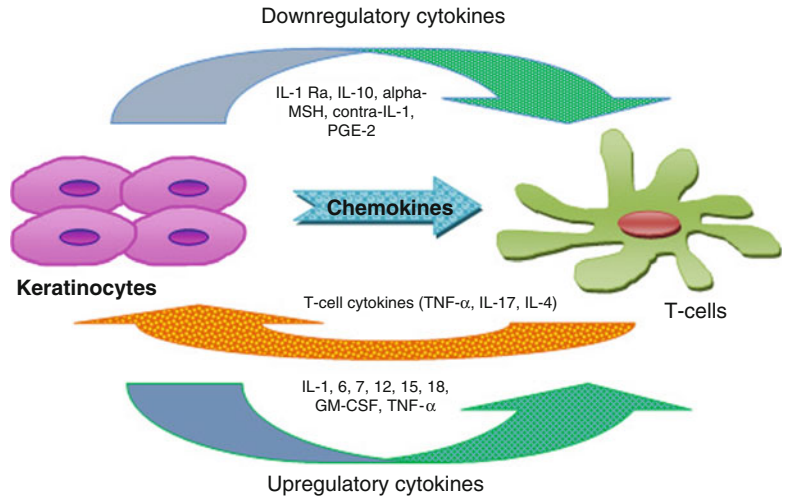

Fig. 21.3 Interrelation mediated by humoral factors between keratinocytes and T cell

capable of eliciting both innate and adaptive immunities (Nestle et al. 2009). The main gate keepers of the skin immune sentinels are DCs, as professional APCs which are capable of eliciting both innate and adaptive immunities. SALT is comprised of LCs, recirculating T lymphocytes, keratinocytes, and a set of draining peripheral lymph nodes. Lymphatics drive the antigens to the lymph nodes where they come in contact with the epidermis and with the APC because migratory T cells are attracted toward the peripheral lymph nodes (Chu et al. 2011) (Fig. 21.3).

21.5 Delivery Considerations

The transport and migration of antigen across the skin barrier, and consequently its uptake and maturation by DCs, are the two main challenges encountered by TCI. Since considerable variation occurs between different species in the structural characterization and lipid composition of the skin, it is imperative to have knowledge of the composition and characteristics of the skin of the species that is to be vaccinated. A minute understanding of the stratum corneum composition is required to facilitate the development of targeted and topically applied vaccine formulation. The highly compact structure of the stratum corneum with alternating hydrophilic and lipophilic area provides effective first-line defense against the invasion of chemicals, pathogens, and therapeutics topically applied to the skin. Alternating hydrophilic and lipophilic areas present a barrier in particular against large and hydrophilic molecules. The purified antigens are highly unstable when applied in their native state, hence innovative strategies are developed (i.e., suitable carrier devices or formulations), which enable antigen stabilization and facilitate their permeation (Combadière and Mahé 2008).

21.5.1 Skin Structure

The skin is composed of three major layers: the epidermis (about 50–150 μm thick), dermis (about 250 μm thick), and hypodermis or subcutis (Young et al. 2006). The stratum corneum being the outermost layer of the epidermis is composed of non-nucleated highly keratinized cells surrounded by densely packed lipid molecules which are responsible for the barrier function of the skin. The use of adjuvants (agents that stimulate the immune system) like bacterial exotoxins, disrupting the stratum corneum by tape stripping, swabbing with alcohol or other solvents, hydration, ultrasound, microneedles, and other physical or chemical permeation enhancers are the general techniques used to overcome the barrier nature of the stratum corneum. These

techniques not only weaken the skin barrier but also activate resident cells to augment expression of cytokine and to enhance antigen presentation. The variations exist in the thickness and composition of the skin at different sites in the human body which affect the permeability characteristics of applied antigens. There are many studies that confirm the difference in the role and responses of skin-resident DCs (Wang et al. 2007), serum antibody responses, and antigen-specific $CD8^+$ cytotoxic T lymphocytes (CTLs) at different anatomical sites.

21.5.2 Transportation into and Across the Skin

Three possible routes via which the antigen can permeate through the skin by a passive diffusion process are transcellular, intercellular, and appendageal routes (Prow et al. 2011). Physicochemical properties such as molecular weight or volume, solubility, and the lipophilicity govern the diffusion rates of the antigen/adjuvants across the intercellular lipidic channels/routes, which have been estimated to be 19 nm. Numbers of approaches have been explored for the efficient delivery of bioactive molecules to the skin, which overcome the barrier properties of the stratum corneum, such as physical, chemical, and vesicular approach (Merwe et al. 2006).

21.5.3 Vesicular Systems for Transcutaneous Immunization

Novel drug delivery systems are being investigated which successfully overcome the problems regarding patient compliance and safety and opened up both opportunities/options for alternative therapeutic strategies to evoke immunological responses without breaching the skin barrier (Teichmann et al. 2007). Moreover, use of these carriers is also beneficial because they require no specially trained personnel and may avoid risk associated with needle-borne prick. Novel vesicular systems could improve vaccination programs by acting as adjuvants to enhance the immunogenicity of antigens, which otherwise induce "weak" immune response when applied topically (Gupta and Vyas 2012). Topical immunization includes the utilization of carriers like liposomes, niosomes, ethosomes, and transferosomes, since they are proficient in transferring immunogens (DNA and antigens) across the intact skin, by enhancing skin permeability for bioactives. These vesicular carriers utilize different pathways in the skin, i.e., either the intercellular lipidic route or the hair follicles to cross the skin barrier and reach the desired cells.

The advantage of using vesicles for vaccine delivery is also their ability to retain the antigen for a longer time and can act as local depot for sustained release of immunogens (Singh et al. 2002).

21.5.3.1 Liposomes

Liposomes are one of the most commonly and extensively studied vesicles representing a promising carrier system for topical delivery of drugs, biologically active molecules, and antigens. They are spherical vesicles made up of phospholipids (varying lipid composition) amenable for cutaneous delivery. Various mechanisms by which the antigen-loaded liposomes permeate through the skin are schematically presented in Fig. 21.4. These mechanisms may be attributed to the similarity in lipid composition of liposomes to the epidermis that enables them to penetrate deeper into the skin through the epidermal barrier. Dermal accumulation and depot formation of drugs/antigens by liposomes are responsible for the localized effect of antigens.

Liposomes encapsulating epitope from *Plasmodium falciparum* were evaluated in a clinical study (Fries et al. 1992), and they were found to be safe and effective as vaccine against malaria. Initially, simple liposomes were used for the introduction of genes (plasmids) and oligonucleotides into cells; however, later modified cationic lipids were used to improve the compaction of the DNA and to neutralize the negative charge.

Advanced types of liposomes like pH-sensitive liposomes may promote the fusion of the membrane of liposome with the cell

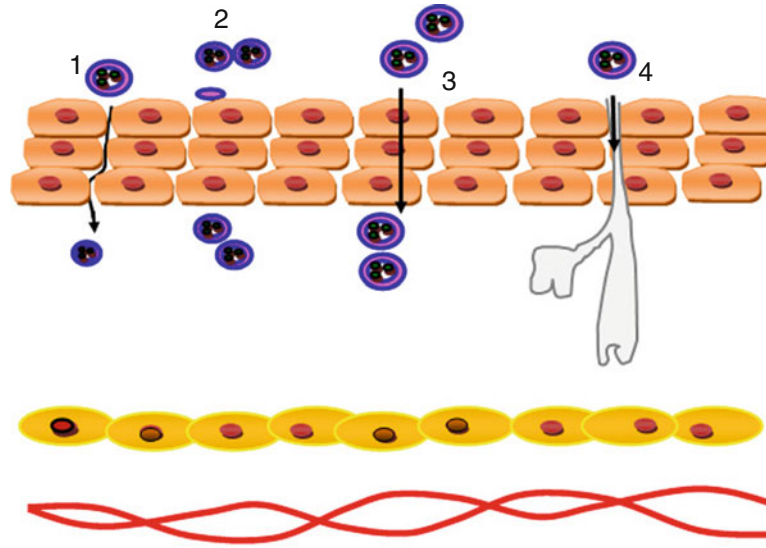

Fig. 21.4 Various mechanisms of penetration of antigen-loaded liposomes across the skin: (*1*) intercellular transport, (*2*) integration with skin lipids, (*3*) transcellular transport, (*4*) pilosebaceous-mediated delivery

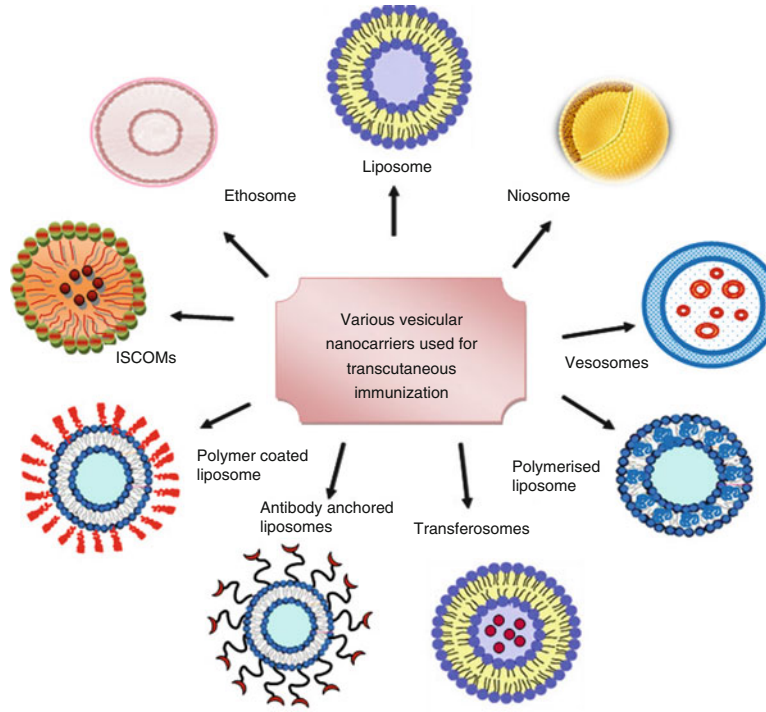

Fig. 21.5 Various vesicular nanocarriers that can be explored for transcutaneous immunization

membrane or within the lysosomal membrane at low pH and thus allow the DNA to escape into the cytoplasm of the cell (Couvreur et al. 1997). Oleic acid, palmitoyl-N-homocysteine, dipalmitoyl succinyl glycerol (DPSG), and cholesteryl hemisuccinate (CHEMS) are the agents that formulate pH-sensitive liposomes. As liposomes were successful in gene therapy trials, they can also be exploited for the delivery of plasmid-encoding genes from pathogens in order to stimulate immunity against that pathogen. Schematic representations of various vesicular carriers that can be used for TCI are shown in Fig. 21.5.

21.5.3.2 Immune-Stimulating Complexes (ISCOMS)

ISCOMs are spherical, micellar matrix constructs of about 40 nm diameter and have size comparable to that of viruses (Cui and Mumper 2002). ISCOMs incorporate amphiphilic antigens like membrane proteins, saponin mixture (Quil A), cholesterol, and phospholipids. These may promote endocytosis of antigens by DCs, monocytes, and macrophages, thus effectively promote T- and B-cell activation (Cui and Mumper 2001).

It was hypothesized that ring-shaped micelles with a diameter of about 10 nm are the building blocks of ISCOMs where the composition influences the aggregation behavior. In the early 1970s, Quil A, a potent adjuvant, has been used as such in veterinary vaccines. Hydrophilicity in the outer area is maintained by the high fraction of Quil A which is essential to prevent micelle–micelle hydrophobic interactions. Fluidity of the micelles is due to phospholipids that allow the formation of spherical structures: i.e., empty ISCOMS consisting of about 14 ringlike micelles. ISCOMs can only contain antigens if hydrophobic; electrostatic interactions or hydrogen bonding (between carbohydrates) is established and involved (Windon et al. 2002).

21.5.3.3 Niosomes

Niosomes are nonionic surfactant-based vesicles that have gained wide acceptance as the topical carrier for dermal or transdermal delivery of bioactives and immunogens (antigens or DNA). As compared to liposomes which cause corneocyte swelling and disruption of the intercellular lipid ultrastructures, niosomes made up of decycloethyleneoleylether result in fusion of corneocytes and formation of lipid stocks. Niosomes can also be used for targeting of immunogens to the pilosebaceous units in order to transfer the immunogens or other active substances to the deeper skin layers. Vesicles also protect antigen from degradation by enzyme attack and hence act as rate-limiting membrane barrier serving as a local depot for the sustained release of encapsulated antigen (Schreier and Boustra 1994). Niosomes of optimum size (2–6 μm) play an important role in the case of pilosebaceous targeting as they enter the pilosebaceous units against the sebum outflow. Drug transport across the skin depends on the vesicle composition and physicochemical properties as it was reported that liquid-state vesicles are more effective than gel-state vesicles in enhancing drug transport (Vyas et al. 2005).

In a study by our group, topical delivery of niosomes containing plasmid DNA encoding hepatitis B surface antigen (HBsAg) was investigated (Vyas et al. 2005). These niosome-based systems were applied topically to mice, and there serum anti-HBsAg titer and cytokine levels (IL-2 and IFN-c) were assessed for the immune-stimulating activity. Titer values obtained after 6 weeks were analogous and comparable to that elicited by intramuscular injection of pure HBsAg. In addition, a high thermodynamic activity gradient is created at the bioactive stratum corneum interface as a result of adsorption and fusion of niosomes onto the surface of the skin (Schreier and Boustra 1994). Surfactants used in the niosome formulation enhance penetration and reduce the barrier property of the stratum corneum (Valjakka-Koskela et al. 1998). Uptake of antigen-loaded nanocarriers by the LCs is represented in Fig. 21.6.

Niosomes containing HBsAg for topical immunization have been prepared by using reverse-phase evaporation technique with an entrapment efficiency of 58.11 ± 0.71 % (Maheshwari et al. 2011). In another study, to target LCs, niosomes were coated with a modified polysaccharide O-palmitoyl mannan (OPM) (Jain and Vyas 2005), and it was resulted that niosomal formulations showed a significantly higher serum immunoglobulin G (IgG) titer than alum-adsorbed BSA ($P < 0.05$) when applied topically. Moreover, it was also found that mannose-coated niosomes elicited appreciably higher serum IgG levels as compared with plain uncoated niosomes ($P < 0.05$).

21.5.3.4 Ethosomes

Ethosomes are interesting and innovative vesicular carriers that are soft and malleable, hence, enabling improved delivery of active agents. They represent ethanol-containing liposomes, which are able to provide an effective antigen

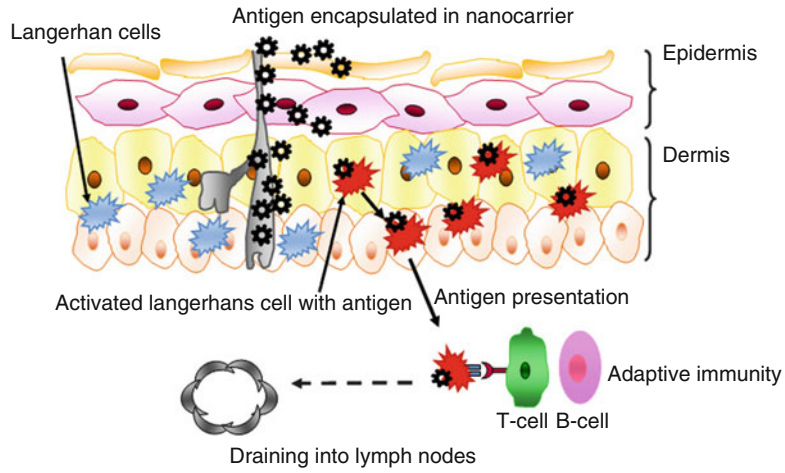

Fig. 21.6 Schematic of nanocarrier systems co-encapsulated with antigen taken up by Langerhans cells for transcutaneous immunization

delivery to deep skin strata more efficiently than conventional liposomes (Dubey et al. 2007; Dayan and Touitou 2000). In a study, a robust systemic and mucosal humoral immune response was elicited when HBsAg-loaded ethosomes were applied topically in experimental mice. In vitro permeation studies using human cadaver skin revealed that transcutaneous delivery of the antigen was much higher for antigen-loaded ethosomes in comparison to antigen-loaded liposomes and plain HBsAg solution. HBsAg-loaded ethosomes are reported to have the ability to carry the antigen(s) to target the immunological environment of the skin and are able to produce a protective immune response. Thus, it was shown that ethosomes possess a great potential in the development of a transcutaneous vaccines (Mishra et al. 2008).

21.5.3.5 Transfersomes

Transfersomes™ (IDEA AG, Germany) are specially designed unique lipid and surfactant-based vesicles that offer flexible characteristics and excellent approach for topical immunization. These ultradeformable carrier systems are highly efficacious in transferring the bioactive molecules across the stratum corneum by virtue of their high capability of changing shape and passing through the natural pores in the skin layer. Transfersomes™ of diameter 500 nm are able to pass through a skin pore of diameter less than 100 nm which clearly indicates that these carriers can permeate through the minute pores present in the skin having a diameter five times less than their own diameter.

After the transfer of antigen-loaded vesicles through the intact skin, antigen is delivered to the lymphatics from where they can be transferred to lymph nodes. It is found that Transfersomes™ with respect to other vesicular carriers give rise to elevated antibody titer. Moreover, when applied topically in a low dose, they show comparable titer values with their intradermally applied counterparts. The Transfersomes™ are under investigation for the development of human vaccines, and if designed suitably, they can have satisfactory immunoadjuvant action and ability to target macrophage. Transfersomes™ incorporating gap junction proteins of bacteria have been developed for the topical application and resulted in higher titer value of antibodies against the gap junction proteins than subcutaneous injection (Paul et al. 1998).

The structural flexibility of the Transfersomes™ is due to the presence of sodium deoxycholate. Cationic lipids being positively charged like DOTMA (N-[1-(2,3-dioleyloxy)-propyl]-N,N,N-trimethylammonium chloride) can be used along with sodium deoxycholate for preparing a novel vesicular construct that has the capability of penetrating intact skin and tends to form a composite complexation with anionic DNA. In a study by our group, the possibility of cationic Transfersomes™ to be used as topical

carriers for DNA vaccine was studied. The specific immunological response induced by plasmid DNA encoding for HBsAg antigen loaded in cationic Transfersomes™ was compared with that elicited by topical or intramuscular administration of liposomes and naked plasmid DNA. DOTMA was used as a cationic lipid, instead of egg PC, with sodium deoxycholate. Under an electron microscope, these cationic vesicles appeared as unilamellar vesicles as shown in Fig. 21.7 (Mahor et al. 2007). Results revealed that immune responses with DNA-loaded cationic Transfersomes™ were considerably higher as compared to naked DNA when mice were topically immunized. Moreover, the antibody titer obtained after 6 weeks was analogous and comparable to that elicited by intramuscular injection of pure HBsAg. DNase enzymes present in interstitial space hydrolyse the naked DNA (Perrie and Gregoriadis 2000); thus, the vesicles in addition to their intrinsic ability to be taken up by the APCs also shield DNA from hydrolytic attack by DNase.

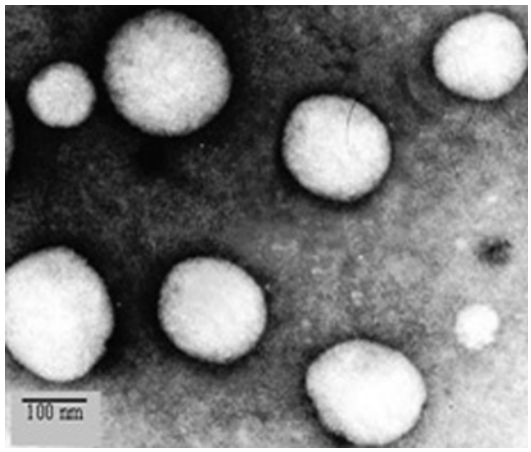

Fig. 21.7 Transmission electron microscopic image of DNA-loaded cationic transfersomes (Mahor et al. 2007)

The immunity induced by topical immunization appears to be long lasting, as indicated by persistence of serum antibodies. Sodium deoxycholate present in the Transfersomes™ is responsible for their deformability, whereas niosomes and liposomes usually contain cholesterol that imparts rigidity to the vesicle. Thus, niosomes and liposomes are not much capable of passing through the pores smaller than their own diameter.

In a study by our group, elastic vesicles Transfersomes™, niosomes, and liposomes were compared for their potential in noninvasive tetanus toxoid (TTx) delivery (Gupta et al. 2005). It was found that Transfersomes™ can entrap higher amounts of proteins as compared to liposomes and niosomes (Yoshioka et al. 1994). Schematic presentation of mechanisms of penetration of antigen-loaded transferosomes across the skin epithelium is shown in Fig. 21.8.

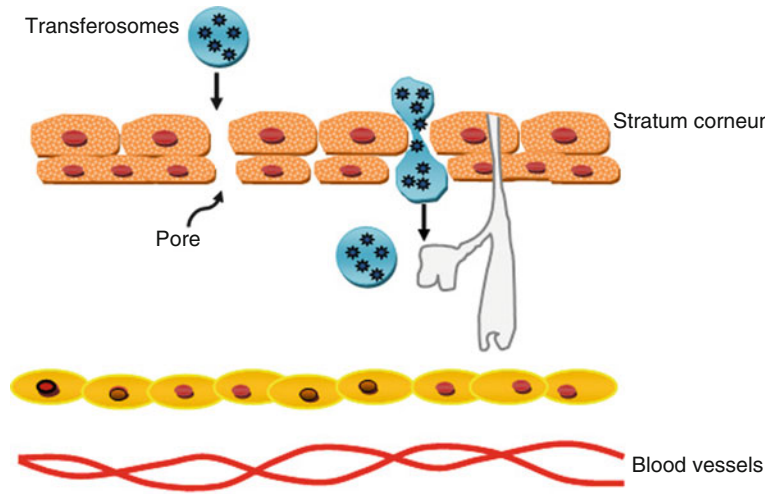

Fig. 21.8 Mechanisms of penetration of antigen-loaded transferosomes across skin epithelium

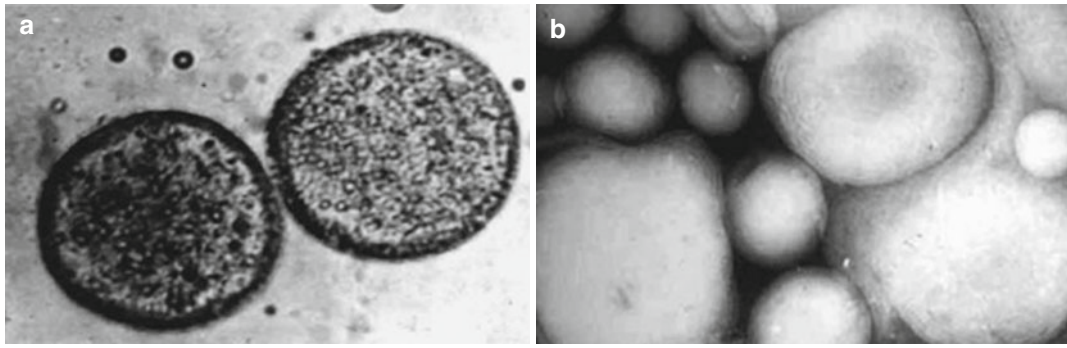

Fig. 21.9 Transmission electron microscopic image of vesosomes (**a**). Phase contrast image of fusogenic vesosomes (**b**) (Adapted from Mishra et al. 2006a, b)

In the case of liposomes and niosomes, the entrapment efficiency was almost equivalent; however, in case of niosomes, slightly less drug entrapment was estimated. This may be due to the presence of surfactants in niosomes that are responsible for the pore formation in the outer layer leading to lower drug entrapment. However, presence of nonionic surfactants in niosomes is responsible for the enhanced permeation effect which is reflected by the better immune response elicited by niosomes than by liposomes. Deformability, a unique property of Transfersomes™, is combined with sensitivity of immunization. Numerous pores in the horny region of the skin may act as permeability shunts and locally lower the skin barrier potential. Transepidermal water gradient strongly drives the deformable Transfersomes™ through these pores. Because of the low deformability of liposomes and niosomes, they are not able to enter the intact skin spontaneously as Transfersomes™. Thus, it can be concluded from the reported studies that Transfersomes™ can be regarded as superior delivery systems for TCI owing to the higher entrapment efficiency and maintenance of better immune response.

21.5.3.6 Vesosomes

Vesosomes, i.e., fusogenic liposomes, are a class of novel carriers which have the unique property of fusing with the target cell and can potentially aid the intracellular delivery of encapsulated antigen/proteins. Lipids, such as dioleoylphosphatidylethanolamine (DOPE), that are able to form non-bilayer phases, contribute in the formation of vesosomes which can promote destabilization of the bilayer of vesicles, inducing fusion events (Kono et al. 2000). Mishra et al. 2006b have studied tetanus toxoid (TTx) containing vesosomes, which can deliver it effectively in order to produce an effective immunization via topical administration. Structural studies on the shape of the prepared vesosomal system using transmission electron microscopy and phase contrast microscopy have indicated that the systems were almost spherical with smooth surface and unilamellar in nature (Fig. 21.9). The developed novel fusogenic vesosomes, composed of inner cationic liposomes contained in an outer liposomal bilayer, have the potential to microinject entrapped antigen directly into the cytoplasm of target cells through fusion with the plasma membrane.

Fusion of vesosomes with the APCs results in cytosolic delivery of the antigen. It is evident by the earlier studies that highly charged particles with size greater than 10 μm are unable to reach deep into hair follicle due to chemical environment present in hair follicle. Moreover, microparticles less than 3 μm are distributed

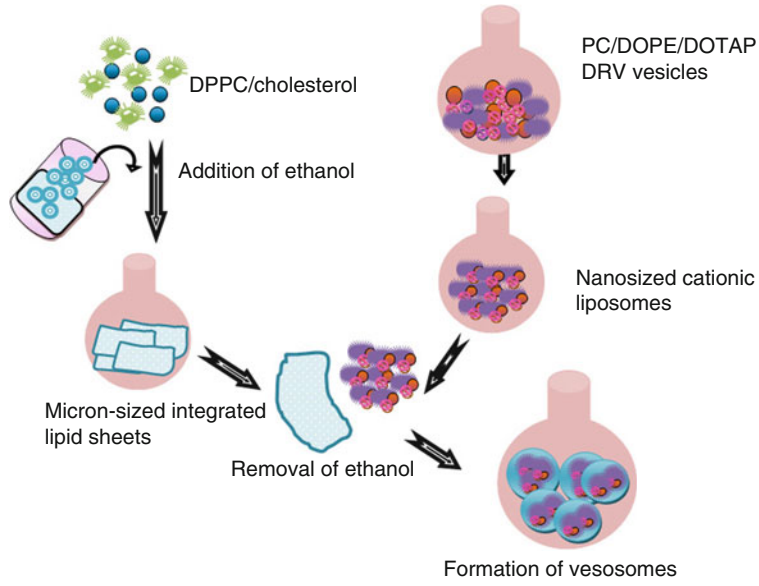

Fig. 21.10 Preparation of vesosomes

randomly into hair follicles and stratum corneum, and those ranging from 3 to 7 μm could selectively penetrate follicular ducts (Rolland et al. 1993). Taking these facts into consideration, vesosomes have been designed and optimized in respect of size and charge. Level of IgG in the skin was increased considerably with vesosomal systems compared to conventional liposomal formulations administered topically. This may be possible due to the release of encapsulated cationic vesicles within hair follicles and subsequently fusion of these vesicles with immune-responsive cells (e.g., LCs, epidermal T cells) for better and more effective antigen presentation. Furthermore, encapsulated antigen may be released through cationic fusogenic liposomes in the vicinity of these cells (Mishra et al. 2006a).

In a study by Baraka et al. 1996, the fusogenic properties of non-phospholipid liposomes containing dioxyethylene acyl ethers and single-tailed non-phospholipid amphiphiles as principal membrane constituents were prepared. These liposomes can fuse with phosphatidylcholine liposomes at neutral pH. Scheme presentation of preparation of vesosomes is shown in Fig. 21.10. Further, studies indicated that these non-phospholipid liposomes could fuse effectively with the plasma membranes of erythrocytes and fibroblasts.

Table 21.1 shows some of the nanocarriers used for the transcutaneous delivery of antigens.

Table 21.1 Schematic overview of nanocarrier-mediated gene/antigen delivery after topical application onto the skin

Delivery system	Vesicle composition	Antigen/DNA/plasmid encoding	Tested on	Effect	Reference
Cationic Transfersomes™	DOTMA and sodium deoxycholate	Hepatitis B surface antigen (HBsAg)	HepG2 cell line has >90 % cells were viable	Higher anti-HBsAg antibody titer and cytokines level compared to pure HBsAg	Mahor et al. (2007)
Cationic liposomes	Soybean phosphatidylcholine (SPC), 1,2-dioleoyl-3-trimethylammonium-propane chloride salt (DOTAP) and 1,2-dioleoyl-sn-glycero-3 phosphoethanolamine (DOPE)	Ovalbumin (OVA) and a toll-like receptor (TLR) ligand	HEK293 cells	Interferon production by restimulated splenocytes, OVA/CpG liposomes shifted the IgG1/IgG2a balance more to the direction of IgG2a	Bal et al. (2011)
Ethosomes	SPC, Span® 80	HBsAg	NIH3T3 cells (embryonic fibroblast cells)	Stimulate the T lymphocytes and generation of TH1-type immune response higher than liposomes and soluble HBsAg	Mishra et al. (2010)
Vesosomes	Phosphatidylcholine, DOPE, DOTAP, dipalmitoyl phosphatidylcholine (DPPC)	Tetanus toxoid (TTx)	Murine macrophage cell line J774 A.1	Serum IgG titer was higher than alum-adsorbed TTx given intramuscularly and topically administered plain tetanus toxoid solution, plain liposomes, and cationic fusogenic liposomes. The vesosomal systems could elicit combined Th1 and Th2 immune responses following topical administration	Mishra et al. (2006b)
Elastic liposomes	SPC was mixed with Span® 80	HBsAg	–	Induces robust systemic and mucosal antibody response, efficient delivery of antigens to LCs cells and lymphatics	Mishra et al. (2006a)

Elastic liposomes	SPC was mixed with Span® 80	HBsAg	Murine bone marrow cells	Induced approximately a two- to threefold increase in IL-2 levels, four- to fivefold increase in IFN-γ levels and twofold increase in TNF-α levels	Mishra et al. (2007)
Niosomes	Span® 80 and cholesterol	HBsAg	–	Serum IgG titers and higher IgG1/IgG2a ratio suggest induction of both Th1 and Th2 responses	Maheshwari et al. (2011)
Novel-modified liposomes (ethosomes)	SPC	HBsAg	Murine bone marrow cells	Predominantly TH1 type of immune response	Mishra et al. (2008)
Niosomes	Sorbitan monostearate/sorbitan trioleate (Span® 60/Span® 85), cholesterol, and stearylamine	Bovine serum albumin (BSA)	–	Significantly higher serum IgG titer upon topical application as compared with topically applied alum-adsorbed BSA, eliciting both humoral and cellular responses	Jain et al. (2005)
Elastic vesicles	Sucrose-laurate ester, octaoxyethylene-laurate ester	Diphtheria toxoid (DT)	–	Surfactant-based vesicles served as adjuvants	Ding et al. (2008)
Deformable liposomes		HBsAg plasmid DNA		Elicited a comparable serum antibody titer and endogenous cytokine levels compared to other vaccines	Wang (2007)
Transfersomes™	DOTAP:NaC	GFP reporter	Mouse skin	GFP expression in the liver and lungs	Kim et al. (2004), Lee et al. (2005)
Hybrid nonionic cationic liposomes	GDL:Chol:POE-10:DOTAP	Human interleukin-1 receptor antagonist (IL-1ra)	Hamster skin	Transgene expression in perifollicular cells	Niemiec et al. (1997)

(continued)

Table 21.1 (continued)

Delivery system	Vesicle composition	Antigen/DNA/plasmid encoding	Tested on	Effect	Reference
Biphasic vesicles	SPC:Chol:DC-Chol SPC:Chol:DMPC	Glycoprotein D (gD)	Mouse skin	Elevated anti-IgD IgG gD-specific cellular response (IL-4) in spleen cells	Babiuk et al. (2002)
Niosomes	Tween®61:Chol: DDAB	Luciferase	Reporter in vitro rat skin	High cumulative amounts in skin and high transdermal fluxes	Manosroi et al. (2009)
Niosomes	GDL:Chol:POE-10	bGr	Rat skin	Intense staining of follicular and epidermal cells	Raghavachari and Fahl (2002)
Niosomes	GDL:Chol:POE-10	IL-1ra	Hamster ears	IL-1ra expression in hair follicles	Ciotti and Weiner (2002)

HepG2 hepatocellular carcinoma, *IL-1ra* human interleukin-1 receptor antagonist, *SPC* soybean phosphatidylcholine, *bGr* b-galactosidase reporter, *DOTMA* N-[1-(2,3-dioleyloxy)-propyl]-N,N,N-trimethylammonium chloride, *Span® 80* sorbitan monooleate, *Tween®61* PEG-4 sorbitan monostearate, *HEK293* human embryonic kidney 293 cells, *GDL* glyceryl dilaurate, *CHOL* cholesterol, *POE-10* polyoxyethylene-10, *DOTAP* N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethylammonium, *GFP* green fluorescent protein, *DDAB* didecyldimethylammonium bromide

Conclusion

Topical immunization appears to be an attractive vaccine delivery strategy that enables the use of a variety of antigens and adjuvants. Noninvasive vaccination has proved to be an efficient option for the successful expression of antigen followed by enhanced immune response, making the subject immunized against the disease. Antigens in conventional delivery systems containing classical penetration enhancers are unable to penetrate through the intact skin. Immunologically rich cutaneous surface containing the immune-responsive cells are responsible to initiate an adaptive immune response. Immunization through cutaneous surfaces takes advantage of the assortment of immune-responsive cells in the skin to initiate an adaptive immune response.

Deeper understanding of the cutaneous cells and the antibody and cell-mediated responses promoted the research for more options for TCI. Both human and murine studies support the use of TCI for the induction of protective systemic and mucosal immune responses. In the recent years, significant researches have explored the immune mechanisms and modes of action of adjuvants and thus made vaccine delivery a well-defined science with a potentially immense medical and economic impact. The knowledge of immune mechanism involved and optimization of administration route, delivery system, immune modulator, and formulation stability led to the development of safer and better vaccines. Clinical research on adjuvants for noninvasive delivery and ex vivo use of human material still has to be unraveled and requires further efforts for further exploration. Moreover, humanized animal models should also be exploited for the understanding and better development of transcutaneous vaccine. Site of vaccine administration, type of pretreatment if any, dosing, and the selection of appropriate adjuvant, concentration, and type of penetration enhancer to be used are many variables to be considered. Noninvasive immunization has shown its applicability and success in multiple mucosal compartments (respiratory, digestive, and female genitourinary tract) making it a promising vaccine delivery strategy for safe and effective immunization against a variety of pathogens.

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Chitosan Nanoparticles in Gene Delivery

22

Julide Akbuga, Suna Ozbas-Turan, and Ceyda Ekentok

Contents

22.1 Introduction

Gene therapy is a remarkable approach for the treatment of a wide range of diseases and is a method to prevent, treat, and modulate genetic and acquired diseases by changing the expression of genes that are responsible for these diseases. It has gained more attention over the past decade. However, the major challenge of this kind of therapy is the development of safe and effective gene carrier systems for delivering DNA to the target tissue followed by internalization since nucleic acids are sensitive to enzymatic attack of nucleases and show poor cellular uptake (Mahato et al. 1999).

At present, there are two classes of gene delivery systems: viral and non-viral systems. Viral gene delivery systems have been largely employed in clinical trials, because of their high transfection efficiency. They efficiently deliver exogenes to host cells by taking advantage of intracellular trafficking. However, several serious drawbacks, including the lack of specificity to target cells; safety concerns, such as risk of potential immunogenicity; and chromosomal insertion of viral genome, and other drawbacks like restricted plasmid size and complicated

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production, limit the practical use of viral vectors. The death of a patient in a gene therapy trial using viral vectors has focused research on non-viral vectors (Glover et al. 2005). Non-viral vectors have attracted much attention as safer alternatives to viral vectors and also due to several advantages, such as ease of production, lower immunogenicity against the repeated administration, well-defined physicochemical properties, unlimited DNA packing capacity, and low cost. Non-viral vectors have a high degree of molecular diversity for making extensive modifications to overcome extra- and intracellular obstacles of DNA delivery (Jeong et al. 2007; Leong et al. 1998). Cationic lipids, liposomes, cationic polymers, and physical methods are used for DNA internalization. Although cationic lipids are easily transferred into the cell and have high transfection efficiency, they are rapidly cleared from the blood, which limits their application (Midoux et al. 2009). They have also some drawbacks such as toxicity and instability (Leong et al. 1998; Borchard 2001). A range of natural and synthetic cationic polymers have also been used, including chitosan, poly(L-lysine) (PLL), poly(ethylenimine) (PEI), protamine, spermine, modified gelatin, poly (β-amino ester), poly (lactide-co-glycolide), and poly (ε-caprolactone), to prepare polymer-DNA nanoparticles for gene delivery (Bhavsar and Amiji 2007). Cationic polymers are more stable than cationic lipids, and they can be condensed with DNA and deliver it to the cells (Hirano and Noishiki 1985; De Smedt et al. 2000).

Among cationic polymers, chitosan is an attractive gene delivery carrier, since it is biocompatible, biodegradable, nontoxic, cheap and has low immunogenicity (Hirano and Noishiki 1985). Chitosan, α-(1–4)2-amino-2-deoxy-β-D-glucan, is a linear cationic polysaccharide prepared by partial alkaline deacetylation of chitin (Skaugrud 1989). Chitin is mostly obtained from exoskeleton of crustacea (shell of shrimp and other crustaceans). Chitosan is a weak base with pKa value of the D-glucosamine residue of about 6.2–7.0 and is therefore insoluble at alkaline and neutral pH value (Fig. 22.1) (Hejazi and Amiji 2003).

The primary amine groups provide special properties to chitosan and make it very useful in pharmaceutical application and gene therapy.

From the technological point, it is very important that chitosan is hydrosoluble and positively charged as these properties are responsible for its interaction with negative charged molecules such as DNA, upon their contact in an aqueous solution.

Chitosan can effectively bind DNA and protect it from the degradation effect of nucleases. Chitosans have different degree of N-acetylation (40–98 %) and molecular weight (50–2000 kDa) (Hejazi and Amiji 2003).

Because of these good properties of chitosan, chitosan nanoparticles have been appealing as a gene delivery carrier in recent years. Chitosan has been used for the first time as a delivery system for DNA by Mumper et al. (1995), and since then chitosan is successfully used as a non-viral gene delivery system both *in vitro* and *in vivo* (Erbacher et al. 1998; MacLaughlin et al. 1998; Roy et al. 1999; Aral et al. 2000; Koping-Hoggard et al. 2001; Ozbas-Turan et al. 2003; Chen et al. 2004).

In gene therapy, transfection is hampered by several problems, including targeting the gene delivery system to the target cell, transport through the membrane of the cells, degradation in endolysosomes, and intracellular trafficking of DNA to the nucleus (Borchard 2001). Chitosan-based gene delivery systems appeared to have the ability to overcome these major obstacles for transfection (Ishii et al. 2001).

In earlier studies, chitosan-DNA was used in many pharmaceutical forms including complexes, self-aggregates, emulsions, microspheres, and nanoparticles (Erbacher et al. 1998; Ozbas-Turan et al. 2003; Lee et al. 1998, 2005a, b; Mao et al. 2001) as a promising gene carrier. On the other hand, gene therapy is based on two basic strategies: (1) gene replacement or correction for the purpose of production of a protein appropriate to a cell function and (2) inactivation of a gene causing cell dysfunction.

In this chapter, transfection efficiency, factors influencing transfection and cellular uptake, and

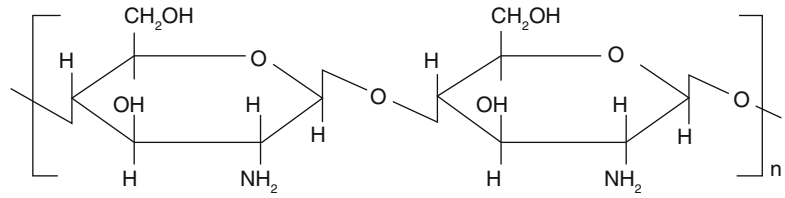

Fig. 22.1 Structure of chitosan

administration routes of chitosan-nucleic acid nanoparticles are discussed.

22.2 Factors Affecting Transfection Efficiency

Transfection is the process of introducing nucleic acids into cells. Different factors may affect the transfection properties of chitosan-based systems. Therefore, optimization of the transfection efficiency of chitosan-DNA nanoparticles is needed for clinical trials. Useful reviews about this topic are available in literature (Kim et al. 2007; Mao et al. 2010) (Fig. 22.2). Some of the factors mentioned below (such as presence of serum and others) are mostly related to parenteral administration, but will be discussed in the chapter as general important data about the gene delivery with chitosan.

22.2.1 Molecular Weight of Chitosan

Molecular weight of chitosan used in gene delivery is an important formulation variable. The effect of molecular weight of chitosan in chitosan-DNA particles has been attributed to chain entanglement effect. Longer polymer chains in high molecular weight chitosan more easily entangle free DNA after initial electrostatic interaction has occurred. Low molecular weight chitosans having shorter polymer chains may not be energetically favorable for chitosan-DNA particle formation (Kiang et al. 2004b). Conflicting results are found in the literature about the influence of chitosan molecular weight on the transfection efficiency. MacLaughlin et al. (1998) studied the transfection efficiency of commercial and prepared depolymerized chitosan (32 and 120 kDa)-DNA complexes on *Cercopithecus aethiops* SV40-transformed kidney cells (Cos-1) and noted that molecular weight of chitosan had limited effect on plasmid expression *in vitro*. Similar observations were reported by several other groups (Koping-Hogard et al. 2003, 2004; Romoren et al. 2003).

However, the group of Sato et al. (2001) investigated factors influencing the transfection efficiency as well as cell transfection mechanisms of chitosan-DNA complexes; the results indicated that chitosans of 10–50 kDa are excellent for gene transfer (Ishii et al. 2001; Sato et al. 2001). According to their suggestion, molecular weight of chitosan may affect the stability, cellular uptake, and the dissociation of DNA from chitosan-DNA complex after endocytosis. A certain combination of deacetylation degree and molecular weight of chitosan is suggested to be an important factor in transfection efficiency by affecting particle stability (Lavertu et al. 2006). Csaba et al. (2009) encapsulated plasmid DNA into chitosan nanoparticles prepared with high and low molecular weight polymers and found that nanoparticles based on low molecular weight chitosan mediated higher plasmid DNA expression than nanoparticles prepared with high molecular weight chitosans.

A charge ratio as high as 60:1 (+/-) was required for the formation of physically stable polyplex when using low molecular weight of chitosan. The degree of polymerization (DPn) of chitosan is also important, chitosan having degree of polymerization around 18 complexed with DNA and transfected *in vitro* and *in vivo* (for lung administration) successfully (Koping-Hoggard

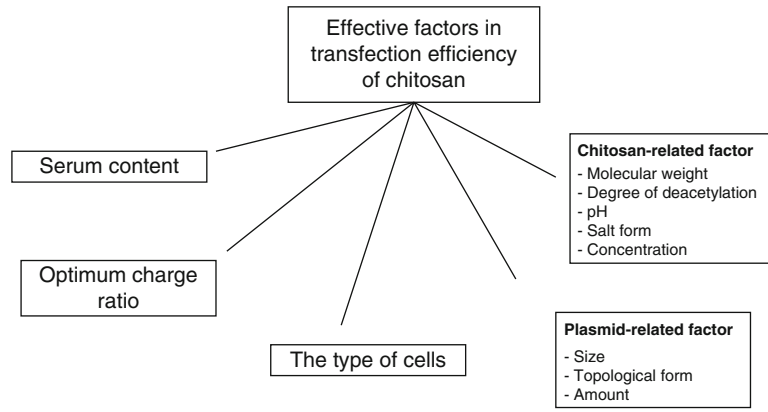

Fig. 22.2 Factors affecting transfection efficiency of chitosan-based gene delivery system

et al. 2004). These polyplexes more easily dissociated and indicated *in vitro* higher transfection than high molecular weight chitosan. *In vitro* transfection requires relatively more stable polyplexes for successful gene transfection than *in vivo* transfection. However, *in vivo* transfection efficiency of chitosan may be dependent on factors other than physical stability and is directly related to the ability of chitosan to protect DNA against the deoxyribonuclease (DNase) enzymatic attack. DNA degradation by DNase is shown to be a major barrier to an efficient *in vivo* transgene expression (in the lung). Therefore, a fine balance must be provided between DNA protection (higher with high molecular weight chitosans) and efficient intracellular unpacking (better with low molecular weight of chitosan) for obtaining high transfection efficiency with chitosans (Koping-Hoggard et al. 2004; Kim et al. 2007).

Akbuga et al. (2004) evaluated interleukin-2 encoding plasmid DNA (pDNA)-loaded chitosan microparticles using low and medium molecular weight chitosan and the effect of molecular weight of chitosan on *in vitro* transfection. The molecular weight of chitosan used and the amount of pDNA influenced the *in vitro* IL-2 production in the cell.

22.2.2 Degree of Deacetylation (DD)

There have been few papers concerning the influence of chitosan properties (other than molecular weight) on gene transfection. In general the degree of chitosan deacetylation greatly affects the characteristics of the chitosan such as charge density (the number of available primary amines for binding), crystallinity, solubility, and degradation rate (Tomihata and Ikada 1997).

Highly deacetylated chitosan (above 80 %) has been generally used to prepare chitosan-DNA complexes (Erbacher et al. 1998; Koping-Hoggard et al. 2001; Mao et al. 2001). In another study Kiang et al. prepared chitosans with various degrees of deacetylation and used in nanoparticle formation. They investigated DNA-binding efficacy, morphology, and gene transfer efficiency of these chitosans (Kiang et al. 2004b). The decreased degree of deacetylation of chitosan (90, 70, and 62 %) resulted in a decrease in luciferase expression levels in human embryonic kidney (HEK 293), human epithelial cervix adenocarcinoma (HeLa), and human squamous cell carcinoma (SW 756) cells. DNA-binding efficacy decreased with decreasing deacetylation degree.

In another study, similar results were reported by Huang et al. (2005). Various degrees of deacetylation (DD 46–88 %) relating chitosan were used in forming chitosan-pEGFP-C2 (Clontech) nanoparticles and cellular uptake and transfection were evaluated in the human lung carcinoma (A549) cell model. Cellular uptake of nanoparticles was significantly reduced by decreasing degree of deacetylation of chitosan. Lavertu et al. (2006) have tried to optimize the effective parameters (mol. wt, DD, and N/P ratio)

related to chitosan. They obtained maximum expression level by using a certain combination of deacetylation degree and molecular weight of chitosans. High expression of DNA could be achieved by simultaneously lowering molecular weight and increasing DD or lowering DD and increasing molecular weight. In other words, the stability of chitosan-DNA systems may be central to describe the effect of parameters (mol. wt, DD, N/P ratio, and transfection medium) on the transgene expression, as complexes that are not sufficiently stable will dissociate early and will show low or no expression. Contrary, the highly stable complexes might not release DNA once inside the cells resulting in low expression. Particle (complex) stability is strongly dependent on electrostatic binding (Lavertu et al. 2006).

22.2.3 Charge Ratio

Electrostatic interaction between chitosan and nucleic acids leads to the spontaneous formation of nanoparticles of different sizes and shapes (Mao et al. 2010). The ability of chitosan nanoparticles to transfect cells efficiently depends on several factors such as the N/P charge ratio represented by the amine group of chitosan-to-phosphate group of DNA or RNA (+/-) ratio used to form nanoparticles. Because only ~90 % of the amino groups in chitosan were protonated at pH 5.5–5.7, an N/P ratio, instead of charge ratio, was used. Large aggregates formed at N/P ratio around 1 but below 0.75 and above 2 yielded submicron size particles. Higher thermal dynamic stability was obtained for nanoparticles prepared with an N/P ratio between 3 and 8 (Mao et al. 2001).

Ishii et al. (2001) noted that the transfection efficiency of the complexes increased at charge ratios of 3 and 5 and decreased at higher charge ratios, due to the relationship between cell uptake of DNA and transfection efficiency.

Similar results were reported by Lavertu et al. (2006). They found that an optimal N/P ratio is needed, because using a low N/P ratio yields physically unstable complexes and poor transfection, while stable complexes prepared at too high N/P ratio can also show decreased transfection as reported previously (Koping-Hoggard et al. 2001, 2003).

22.2.4 pH

The transfection efficiency of chitosan nanoplexes[1] is dependent on the pH of the culture medium because the charge density of chitosan changes with the pH of the buffer. At pH 5.5–5.7, about 90 % of the amino groups of chitosan are protonated (Mao et al. 2001). Binding capacity of chitosan with negative charged DNA increases as pH of medium decreases (Sato et al. 2001). At neutral pH, the degree of protonation decreases; this means that actual charge of the chitosan polyplex[2] is different from the charge of chitosan-DNA complexes of the same charge value made at pH 5.5 (Romoren et al. 2003).

Sato et al. (2001) compared the transfection efficiency of luciferase reporter vector (pGL3)/chitosan complexes in tumor cells between pH 6.9 and 7.6, and the results showed that transfection efficiency at pH 6.9 was higher than that at pH 7.6 because complexes at pH 6.9 are positively charged and easily bind with the negatively charged cells through electrostatic interaction. Similar data were reported by Koping-Hogard et al. (2003), who investigated the influences of pH (2.5–6.5) on the shape of complexes and observed coil-globular-shaped complexes at pH 6.5.

The fraction of soluble globules (globular-shaped complexes) increased as the pH decreased from 6.5 to 3.5, and further acidification decreased the solubility of globules which eventually precipitated at pH 2.5. Different pH values of transfection media, in the range of 6.5–7.4, were tested by Lavertu et al. (2006). Comparable numbers of transfected cells (HEK 293) were found for pH 6.5 and 7.1, while drastically low-

[1] Particle-based systems can be defined on their main groups: involving the combination of DNA with cationic polymers (polyplex), submicron colloidal systems (nanoplex), or lipids (lipoplex).

[2] See Footnote 1.

ered transfection was observed at pH 7.4. They reported that the decrease of medium pH value increases the surface charge of the chitosan-DNA nanoparticles and increases chitosan binding affinity to DNA; thus, complexes are more stable and more efficient in slightly acidic medium. Similar results were reported by different groups (Zhaou et al. 2006; Liu et al. 2005). Chitosan-DNA polyplexes are shown to be very stable under acidic and low ionic strength conditions; however, after changing to physiological conditions, they tend to form aggregates and indicate a decreased binding affinity for DNA with neutral surface charge of polyplexes (Strand et al. 2005).

22.2.5 Presence of Serum

One of the problems for *in vivo* gene delivery mediated by cationic liposomes and lipids is that gene expression is inhibited by serum (Goldman et al. 1997). The development of gene delivery systems that are stable in serum is very important for the improvement of gene therapy by non-viral vector. Sato et al. (2001) investigated the effect of serum on the transfection efficiency of chitosan complexes. The results indicated that the presence of serum enhanced the gene transfer efficiency about 2–3 times than without serum. This effect may be caused by increasing cell function (viability, membrane permeability) in the presence of serum. However, the addition of 50 % fetal bovine serum (FBS) to transfection medium resulted in the decrease of transfection efficiency of chitosan complexes because of cell damage induced by the addition of high amount of serum.

Erbacher et al. (1998) reported that chitosan-DNA complexes more efficiently transfected human epithelial cervix adenocarcinoma (HeLa) cells in the presence of 10 % serum than in its absence.

22.2.6 Plasmid Concentration

The amount of plasmid DNA incorporated within the particles (complexes) plays an important role in the efficiency of transfection process. The transfection efficiency increases with plasmid DNA concentration up to a critical point; thereafter, the transfection keeps constant or decreases significantly (Mao et al. 2010; Romoren et al. 2003).

MacLauglin et al. (1998) used complexes containing 25–400 μg of plasmid, and a plasmid concentration of 100 μg was selected and formed adequate complexes. The results indicated that as the plasmid concentration increased, the diameter of complexes increased. A higher increase in size was observed by increasing DNA concentration and using a higher molecular weight chitosan (102 kDa) than a lower molecular weight chitosan (32 kDa). Thus, formulating complexes of a specific diameter is possible by adjusting plasmid concentration.

Romeron et al. (2003) reported that the concentration of DNA is an important factor for the magnitude of expressed luciferase in the epithelioma papulosum cyprini cells (EPC).

Increasing the DNA concentration from 0.5 to 2.5 μg per well leads to an increase in gene expression (normal dose-response phenomenon). Saturation in the expression levels was observed by a further increase in plasmid concentration to 5 μg per well (Romoren et al. 2003). Similarly, Zhaou et al. (2006) showed that transfection level increased as the plasmid dose increased (0–8 μg/well) in chondrocytes; however, at higher plasmid dose (16 and 32 μg/well), transfection efficiency greatly decreased. This decrease is attributed to the aggregation of chitosan nanoparticles, resulting in lower cellular uptake.

In chitosan-tripolyphosphate nanoparticles, the effect of plasmid DNA loading (5, 10, and 20 %) on the transfection efficiency of nanoparticles was investigated, and 0.5, 1, and 2 μg pDNA doses were used. Based on the results obtained, a dose of 1 μg was chosen for *in vitro* studies (Csaba et al. 2009).

Plasmid encoding interleukin-4 (pIL-4) gene-loaded chitosan-TPP nanoparticles were prepared using 3 different concentrations of plasmid. Encapsulation efficiency, particle size, and transfection efficiency were influenced by the amount of DNA. Nanoparticles formulated with high

amount of DNA showed the highest IL-4 production (Ozdemir et al. 2011).

22.2.7 Chitosan Salt Form

Chitosan is only soluble in acidic solutions, but its salt forms are water soluble and have improved transfection efficiency. Weecharangsan et al. (2008) studied polyplexes formulated with different chitosan salts such as hydrochloride, acetate, lactate, aspartate, and glutamate and found that the transfection efficiency was dependent on the salt form.

22.2.8 Chitosan Concentration

In addition to abovementioned factors, concentration of chitosan was investigated as a parameter influencing the transfection efficiency. Stable and uniform nanoparticles were formed with chitosan concentration in the range from 50 to 400 μg/mL of chitosan. After i.m. injection of pDNA-loaded chitosan microspheres in rats, a clear effect of chitosan concentration on gene expression was not seen (Aral et al. 2000).

Plasmid IL-4 was successfully encapsulated into chitosan-TPP nanoparticles prepared with different chitosan concentrations (0.25, 0.125 %). Size of nanoparticles changed with the concentration of chitosan. Transfection levels of pIL-4 nanoparticles were also affected by the chitosan concentration, i.e., they were higher when lower concentration of chitosan and higher concentration of DNA were used (Ozdemir et al. 2011).

22.2.9 Cell Type

Chitosan-mediated DNA transfection depends on the cell type; therefore, it is needed to test a gene carrier on different cell lines especially cells that resemble those that will be targeted (Leong et al. 1998; Mao et al. 2001; Corsi et al. 2003). Mao et al. (2001) reported preparation of chitosan-DNA nanoparticles and cell dependency of the transfection efficiency of these particles. Higher gene expression levels were found in human embryonic kidney (HEK 293) cells and bronchial epithelia cells (IB-3-1) compared with that in human tracheal epithelial cell line (9HTEo) and HeLa cells (Mao et al. 2001). Cellular membrane composition varies among the cell types and can facilitate or hinder the binding of the particles and internalization.

In another study, Corsi et al. (2003) evaluated the transfection potential of chitosan-DNA nanoparticles using three different cell lines such as human mesenchymal stem cells (MSCs), HEK 293, and human osteosarcoma cells (MG63) and compared them with Lipofectamine® 2000 (commercial cationic lipid) (Life Technologies). The transfection of HEK 293 cells is superior to that seen with MG63 cells and MSCs. Their results suggested that transfection efficiency of chitosan nanoparticles is depended on the cell type.

22.2.10 Plasmid-Related Factors

Although the role of different factors in transfection was investigated largely, the effect of plasmid-related properties on gene transfection was not studied detailed. The group of Akbuga et al. (2003) investigated the effect of different sized (small and large) plasmids on transfection, and similar transfection data were obtained with two different sizes of plasmids after *in vivo* application to rats. They injected chitosan microspheres containing different forms of plasmid (pMK3) into the muscles of the rats and monitored the transfection profile over 12 weeks. Higher protein production was obtained with microspheres containing a mixture of super coiled and open circular forms (60:40), while the linear form induced lower protein production. Transfectivity of relaxed (a topological form of DNA) or super coiled forms of this reporter plasmid was nearly equal (Akbuga et al. 2003). After co-encapsulation of two plasmids into the same microsphere structure, *in vivo* transfection efficiency was investigated. Plasmid DNAs were continuously released from chitosan microspheres

after their i.m injection to rats, and high β-galactosidase and luciferase productions were determined after a long post-transfectional period (Ozbas-Turan et al. 2003).

22.3 Preparation Techniques of Chitosan Nanoparticles

Chitosan nanoparticles are prepared by coacervation and ionic gelation using sodium tripolyphosphate. DNA is encapsulated into the chitosan nanoparticles or is adsorbed onto the surface of nanoparticles. Another preparation method is the direct formation of nanoparticles from DNA and chitosan by complexation (Fig. 22.3).

22.3.1 Coacervation

Chitosan-DNA nanoparticles were prepared by mixing equal volume of chitosan solution (pH 5.5) and sodium sulfate solution containing DNA (Mao et al. 2001).

Preparation of chitosan microspheres by this method was first described by Berthold et al. (1996). Interleukin-2 (IL-2) expression plasmid (pCXWN-hIL-2)-loaded chitosan microspheres were evaluated using a coacervation preparation method that reported previously by Akbuga et al. (2004) and Aral et al. (2000). IL-2 gene encapsulation was found high (82–92 %). High level of IL-2 expression was measured in MAT- LyLu, the rat prostate adenocarcinoma cell line (Akbuga et al. 2004). Kiang et al. (2004b) prepared chitosan-DNA nanoparticles using coacervation method and reported that degree of chitosan deacetylation is an important factor in gene transfection efficiency *in vitro* and *in vivo*.

22.3.2 Ionic Gelation

This method relies on the interaction between positively charged chitosan and negatively charged polyanion. Nanoparticle formation occurs spontaneously due to the molecular linkages between positively and negatively charged agents. First time Bodmeier et al. (1989) reported ionotropic gelation of chitosan with tripolyphosphate (TPP) for drug encapsulation; however, their approach was to design bead rather than nanoparticle. Then Calvo et al. (1997) have developed chitosan particles based on the same principles.

Chitosan particles with different characteristics can be obtained by changing concentrations of chitosan, TPP, and relative volumes of phases.

Particle size of nanoparticles prepared by this method changed between 93 and 336 nm dependent on molecular weight of chitosan. Nanoparticles showed high DNA encapsulation (almost 100 %) independent of molecular weight and a very well-defined spherical shape (Csaba et al. 2009).

22.3.3 Simple Complexation

Nanoplexes were prepared by mixing a solution of chitosan with nucleic acid solutions. Particle sizes change between 150 and 500 nm. This technique is simple and mild. Particle size depends on molecular weight of chitosan used. The zeta potential of nanoparticle is affected by changing pH and deacetylation degree of chitosan (Lavertu et al. 2006; Huang et al. 2004).

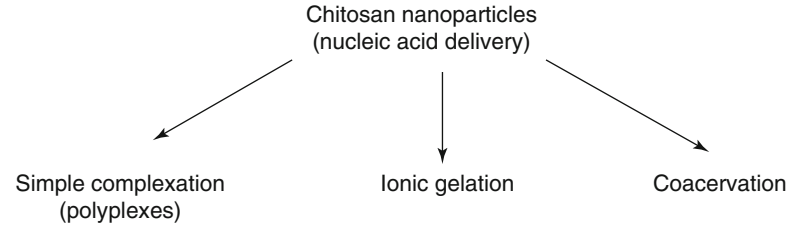

Fig. 22.3 Schematic representation of the preparation method of chitosan-DNA nanoparticles

22.3.4 Nucleic Acid Loading into Nanoparticles

DNA loading in nanoparticles can be done by two techniques, during the preparation of nanoparticles (incorporation) or adsorption after the formation of particles (incubation). In these systems, DNA is physically encapsulated into the matrix or adsorbed onto the surface.

22.4 Modified Chitosans

Despite the several advantages of chitosan as a gene delivery carrier, the application of chitosan/nucleic acid systems is restricted by low transfection efficiency originating from its low solubility and low stability at physiological pH and its slow endosomal release (Mao et al. 2010; Wang et al. 2011). For that reason, several strategies are taken to address these challenges, and hydrophobic and hydrophilic modifications were made to obtain proper chitosan derivatives. Mentioned below modifications of chitosan affecting skin delivery also will be discussed as general important data about other *in vitro* and *in vivo* studies.

22.4.1 Hydrophilic Modification

Positively charged chitosan-DNA complexes can form aggregates following interaction with blood components such as negatively charged serum albumin and other opsonins, resulting in rapid clearance and short circulation time (Dash et al. 1999). In a series of hydrophilic modifications, such as quaternization (Thanou et al. 2002) was applied to enhance transfection efficiency leading to increased water solubility of chitosan at physiological pH, reduced opsonization of the chitosan polyplex, and improved intracellular plasmid release (Mao et al. 2010).

As being hydrophilic, flexible polymer polyethylene glycol (PEG) has long circulating properties, which make it an attractive polymer for modifying the carriers to increase their hydrophilicity and serum half-life (Veronese and Pasut 2005).

22.4.2 Hydrophobic Modification

Hydrophobic modification could enhance cell binding, alleviate serum inhibition, protect from enzymatic degradation, and facilitate intracellular plasmid DNA association, which have been proven to mediate favorable gene transfection (Liu et al. 2010). Hydrophobic modifications of chitosan such as deoxycholic acid (Chae et al. 2005), N-alkylation (Liu et al. 2003), thiolation (Lee et al. 2007), stearic acid (Hu et al. 2006), and uronic acid (Kim et al. 2003) were studied.

N-alkylation is an example of functional group modification of chitosan. The first proposed alkylated chitosan (ACS) was prepared from dodecyl bromide and chitosan (Liu et al. 2001).

Chitosan was hydrophobically modified by deoxycholic acid to yield self-aggregates in aqueous media. These chitosan-deoxycholic acid self-aggregates were used as a delivery carrier for plasmid DNA in mammalian cells (COS-1 cell line). Self-aggregates have a small size with unimodal size distribution (Lee et al. 1998).

Yoo et al. (2005) developed self-assembled nanoparticles using a hydrophobically modified glycol chitosan for gene delivery.

The derivation of the primary amino groups of chitosan with coupling reagents bearing thiol functions leads to the formation of thiolated chitosans. Three types of thiolated chitosans have been developed: chitosan-cysteine conjugates, chitosan-thioglycolic acid conjugates, and chitosan-4-thio-butyl-amidine conjugates. Various properties of chitosan are improved by thiolation: (1) permeation through intestinal mucosa can be enhanced, (2) display *in situ* gelling property, and (3) a prolonged controlled release of embedded molecules (Bernkop-Schnurch et al. 2004). Lee et al. (2007) prepared a highly effective gene delivery system using a 33 kDa thiol-modified chitosan.

22.4.3 Amphiphilic Derivatives

To obtain favorable characteristics for gene delivery, hydrophilic and hydrophobic modifications have been applied to chitosan (CS).

Amphiphilic linoleic acid (LA) and poly (β-malic acid) (PMLA)-double grafted chitosan (LMC)/pDNA nanocomplexes were prepared and characterized. Hydrophobic LA and hydrophilic PMLA substitutions suppressed nonspecific adsorption and enhanced pDNA dissociation. However, enzymatic stability and cellular uptake were promoted by hydrophobic LA grafting. *In vitro* transfection increased 8.0-fold. Higher i.m. gene expression in mice compared to chitosan was reported (Wang et al. 2011).

Transfection activities of N-imidazolys-O-carboxymethyl chitosan/pDNA complexes were studied in HEK 293 cells. High transfection efficiency which is dependent on the degree of imidazoyls substitution was obtained. This result may be due to its high solubility, high DNA-binding capability, and low cytotoxicity (Shi et al. 2012).

22.4.4 Specific Ligand Modification (Active Targeting)

Successful therapy cannot be guarantee if the active molecule does not reach the target site of cell. In spite of the advantages of chitosan as a gene delivery vector, the application and transfection efficiency are limited by low cell specificity, and cellular uptake of nanoparticles mostly occurs via nonspecific adsorptive endocytosis depending on surface properties of chitosan nanoparticles (Mao et al. 2010; Park et al. 2010). Therefore, for improving cellular uptake efficiency and specificity to the target cells, chitosan-based systems are modified by conjugating a cell-specific ligand that specifically recognizes and binds to membrane-bound proteins of target cells. Particularly, cancer cells often overexpress some specific antigens or receptors on their surfaces, which can be used as targets in drug development. The specific ligand-receptor interaction leads to cellular uptake of the chitosan/nucleic acid systems via receptor-mediated endocytosis.

Several targeting molecules and ligands (such as transferrin, folate, galactose, and mannose) have been used for receptor-mediated chitosan modifications.

22.4.5 Other Modification Approaches

One of the important causes of low transfection efficiency of chitosan-based system in the intracellular surrounding is the inefficient release of the nucleic acid of chitosan-DNA complexes from endosomes into the cytoplasm. Therefore, better transfection strategies are needed. For improvement in the endosomal escape capability of the chitosan, imidazole moieties – as a pH-sensitive group – are introduced into the chitosan backbone to mimic the action of PEI (Moreira et al. 2009). For enhancement of endosomal escape, histidine-modified chitosan was reported by Chang et al. (2010). This effect may be due to high buffering capacity of histidine even if introducing very small amount (3–4 %).

Various studies were made on grafting low MW PEI to chitosan in order to enhance the buffering capacity of chitosan, also lowering the cytotoxicity of PEI. PEIs with low molecular weight are nontoxic but have poor transfection efficiency. Branched PEIs with high molecular weight have better DNA transfer ability but high toxicity.

Kiang et al. (2004a) added poly (propyl acrylic acid) (PPAA) in the chitosan-DNA complexes for enhancing the transfection efficiency, because PPAA, a highly pH-sensitive polymer, exhibits maximum membrane disruption capability at below pH 6.0 and results in the disruption of the endosomal membrane to release the vesicle contents into the cytosol. Addition of PPAA to chitosan-DNA complexes enhanced gene expression in both HEK 293 and HeLa cells.

22.4.6 Use of Cell-Penetrating Peptides

The cellular plasma membrane constitutes are effective barrier for many macromolecules

(Bolhassani 2011). Cell-penetrating peptides are short amphipathic and cationic peptides that are rapidly internalized across cell membranes. Some peptide sequences known as protein transduction domains (PTD) or membrane translocalization signals (MTS) were used for the delivery of plasmid DNA (Tung and Weissleder 2003).

With the addition of nuclear localization signal peptide to chitosan-DNA complexes, high gene expression was obtained with the negligible cytotoxicity (Opanasopit et al. 2009).

22.5 Skin Delivery

The skin is an attractive target for the therapeutic and prophylactic gene medicines (containing nucleic acids such as DNA, siRNA, shRNA, and carrier). It is not only the largest human organ but also a good biological barrier to the absorption of drugs and foreign compounds. However, different penetration pathways including hair follicles, sweat ducts, sebaceous glands, and two stratum corneum penetration pathways, the inter cell clusters and the intercorneocyte clusters (being better sealed and more transport resistant), are known, which allow the penetration of exogenous substances into the skin (Cevc and Ulrich 2010). Controllable and reliable molecule delivery across the skin barrier can be provided with stable and deformable nano-sized carriers. The skin has been recently investigated for plasmid DNA delivery as an alternative to parenteral administration of DNA. However, the medical use of gun technology or the needle-free devices are very limited (Cui and Mumper 2001). For achieving local and systemic effects of drugs, the skin is a potential route for drug delivery by nanoparticles. Cui and Mumper (2001) developed chitosan-based nanoparticles (200–300 nm) for topical immunization. They prepared two types of nanoparticles, pDNA-condensed chitosan nanoparticles and pDNA coated on chitosan- carboxymethylcellulose (CMC) nanoparticles, and showed that both chitosan and depolymerized chitosan oligomer can complex CMC to form stable nanoparticles. Plasmid DNA was coated on these pre-formed nanoparticles. Several different chitosan-based nanoparticles containing pDNA were applied topically to the skin of mice. These nanoparticles showed an enhanced luciferase expression in the skin 24 h after topical application. Beside this, for immune response, significant antigen-specific IgG titer was measured as a determinant for expressed β-galactosidase after 28 days of application. Ozbas-Turan and Akbuga (2011) investigated *in vitro* and *in vivo* skin gene transfer of DNA-loaded chitosan/TPP nanoparticles using plasmid that has SV 40 promoter encoding β-galactosidase (pSV-β-galactosidase) (Promega, Madison, WI) as a reporter gene. *In vitro* transfection studies [*Mus musculus* embryo fibroblast cell line (NIH/3 T3) and human dermal fibroblast (HDF)] have revealed that chitosan/TPP nanoparticles are suitable delivery systems for DNA. Lower β-galactosidase level was measured in HDF than in 3 T3 cell lines. In animal studies, usability of nanoparticles was tested and compared in baby and adult rats because of the differences. In baby rats, the hair follicles are virtually in the anagen phase so that the size of the anagen follicles is enlarged and the cells grow in a synchronous manner (Raghavachari and Fahl 2002). Therefore, nanoparticles were tested in both adult and baby rats. Higher gene expression was measured in baby rats than in adult rats. On the other hand, contradictory results were obtained concerning the charge of nanoparticles applied to the skin (Cui and Mumper 2001; Shi et al. 1999). While Cui and Mumper (2001) reported that negative charged nanoparticles achieved higher gene expression, Shi et al. (1999) and Fan et al. (1999) demonstrated the feasibility of cationic particles to be used for topical gene immunization. The results of Ozbas-Turan and Akbuga (2011) were in accordance with the results of Shi et al. (1999). According to histological data of Ozbas-Turan and Akbuga (2011), β-gal expression was mainly localized in the dermis and hypodermis particularly close to the hair follicles. Badea

et al. (2007) used cationic nanoparticles as a topical formulation for the skin delivery of interferon gamma.

Gene guns have been used for the delivery of nucleic acid-coated gold particles through the stratum corneum to the epidermis by helium. Using the gene gun, coated DNA can be inserted into the cytoplasm and nuclei of cells enhancing expression of the encoded protein. However, non-biodegradable gold particles may cause adverse side effects when accumulated (Lin et al. 2006, 2008). Instead of gold particles, biodegradable nanoparticles composed of chitosan and poly-γ-glutamic acid were prepared by an ionic-gelation method for transdermal DNA delivery and used with a low-pressure gene gun. Chitosan-DNA nanoparticles are compared with nanoparticles containing CS/γ-PGA (poly-γ-glutamic acid)/DNA, provided an enhanced penetration depth of DNA into the mice skin and increased gene expression. Lee et al. suggested that this enhancing effect may be due to the fact that chitosan-poly-γ-glutamic acid/DNA nanoparticles were more densely arranged (showing very close packing) than chitosan nanoparticles, thus having a larger mobility for penetration nanoparticles into the skin barrier (Lee et al. 2008).

Chitosan exhibits outstanding properties, such as having a protonable amino group for complexation with nucleic acids, mucoadhesive properties, and permeation-enhancing ability. These remarkable properties of chitosan offered opportunities for its biomedical application (Croisier and Jerome 2013). In burn treatment different types of skin substitutes, i.e., epidermal equivalents, dermal equivalents, and composite equivalents, have been used (Pereira et al. 2007). However, the major limitation of dermal equivalents for treatment of deep burn is slow vascularization, which may result in graft necrosis. To enhance angiogenesis plasmid DNA encoding vascular endothelial growth factor-165 (VEGF-165) was complexed with N,N,N-trimethyl chitosan chloride (TMC) and loaded into bilayer porous collagen-chitosan/silicon membrane dermal equivalents (BDE). These skin substitutes were applied for the treatment of full-thickness burn wounds in the skin. Different BDEs were then transplanted in porcine full-thickness burn wounds. After treatment the TMC/pDNA group had a higher number of newly formed mature blood vessels and faster regeneration of the dermis compared to control groups. After 14 days, a further ultrathin skin grafting was observed on the regenerated dermis, leading to complete regeneration of the skin of the burn wound (Guo et al. 2010, 2011).

Skin is a highly immune-reactive organ containing antigen-presenting cells such as Langerhans cells (LCs), particularly in the epidermis, and it provides a favorable site for DNA vaccines (Lee et al. 2010). Multifunctional core-shell polymeric nanoparticles composed of PLGA-core and glycol chitosan-shell were prepared and applied into the epidermis via a gene gun. Nanoparticles transfected DNA directly into LCs present in the epidermis. Transfected LCs then migrated to lymph nodes and expressed the encoded gene products in the skin draining lymph nodes (Lee et al. 2010). Here, LCs may be activated by antigens in the periphery and move to the lymphoid organs to stimulate an immune response.

Salva et al. (2011) evaluated chitosan/pGM-CSF (plasmid encoding granulocyte macrophage colony-stimulating factor) complexes *in vitro* and suggested that this system may be useful for wound healing.

Conclusions

The skin represents a site for the treatment of cutaneous diseases as well as systemic diseases. Methods for gene delivery via skin have been developed as a therapeutic strategy for the treatment of different skin disorders. Both viral and non-viral methods have been studied. However, due to safety concerns, the use of viral methods is being questioned and non-viral alternatives are gaining major interest. Chitosan-based gene delivery by topical DNA application onto the skin has great potential.

Although important development was achieved in chitosan-based systems for gene delivery, the therapeutic effectiveness still requires to be improved for clinical administration. Standardization of chitosan for

pharmaceutical use, regarding solubility, *in vivo* stability, cell uptake, and unpacking, is an essential problem that must be solved. Also more *in vivo* studies are needed to be carried out in order to overcome the hurdles related to skin delivery of chitosan particles.

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Gene Suppression with Chitosan Nanoparticles

23

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Contents

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23.1 Introduction

Antisense oligonucleotides (ASOs) and small interfering RNA (siRNA) are quite effective in decreasing or inhibiting gene expression and protein production, a process known as "gene knockdown" or "gene silencing." Both of them provide specific and efficient gene silencing, and both are useful tools to study gene function. ASO and RNAi technologies share many problems such as the difficulty of transfection in different cell types, instability in *in vivo* conditions, and site-selective (specific) toxicity at high concentration.

In 1998, two antisense oligonucleotides, Vitravene® (fomivirsen) and Macugen® (aptamer ASO), were approved by FDA for the treatment against cytomegalovirus-induced retinitis by intraocular injection. Recently, Kynamro® (mipomersen, subcutaneous injection) has received US regulatory approval for the treatment of homozygous familial hypercholesterolemia (Crooke 1998; Rayburn and Zhang 2008). Hundreds of ASOs have been examined in preclinical and preliminary clinical trials and many have shown significant activity. There have been performed clinical trials for more than 30 ASOs. While many of the ASOs did not past Phase I trial, several have been in advanced trials (Rayburn and Zhang 2008).

Aim of the antisense therapy is to inhibit the existing but abnormally expressed genes by blocking the replication or transcription of DNA

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or arresting the translation of mRNA to protein (Schreier 1994).

ASOs and siRNAs can also be encapsulated into chitosan matrix using conventional methods such as ionic gelation and coacervation for nanoparticle preparation. In addition to these techniques, nanoparticles can be spontaneously formed by the interaction of positively charged chitosan with negatively charged nucleic acids.

Similar to DNA-chitosan nanoparticles (see Chapter 22, Vol. 1), molecular weight of chitosan affects the physicochemical and transfectional properties of chitosan nanoparticles containing ASO/RNAi-based therapeutics. Moreover, degrees of chitosan deacetylation, pH of the system, and methods of nucleic acid association to nanoparticles have an import role in transfection efficiency of chitosan nanoparticles containing antigene molecules.

In this chapter, different oligonucleotides (siRNA, ASO) with different delivery systems will be mentioned about skin delivery, and also general important points will be discussed about these topics.

23.2 Chitosan/Antisense Oligonucleotides Nanoparticles

The antigene strategy is based on short, synthetic ASOs to form triple helical structures with DNA to interfere with gene expression at the level of transcription (Akhtar 1998). ASOs can be used to specifically inhibit gene expression. These molecules of usually 15–20 bases length can induce Watson-Crick hybridization to target mRNA and via a variety of mechanism inhibit translation of mRNA into protein (Lebedeva et al. 2000). ASOs are anionic and labile macromolecules that rapidly degraded by nucleases. These molecules cannot rapidly move through the cell membranes (Weyermann et al. 2004). They show poor bioavailability; therefore, it is needed to develop suitable ASO delivery system. To improve their transfection efficiency and bioavailability, two different approaches are available: one of them is the preparation of various types of chemically modified ASOs such as phosphorothioate oligodeoxy nucleotide (PS-ASO), mixed backbone oligonucleotides, and morpholine oligonucleotides, and the other is the delivery of ASOs into cells with the help of a suitable carrier (Akhtar et al. 2000). In general, the same gene delivery systems are used as carriers for ASOs. The use of particulate carriers such as liposomes or nanoparticles may be a more realistic approach to deliver ASOs (Lambert et al. 2001). There have been several reviews that provide good overviews of chemical modifications of ASOs (Crooke 2004) and delivery systems for them (Weyermann et al. 2004; Lambert et al. 2001). Among the cationic polymers, chitosan is a good carrier for ASOs because of its positive surface charge, biodegradability, biocompatibility, and low toxicity. Calvo et al. (1998) indicated that ASO was more efficiently associated when the nanoparticles were formed by ionic gelation of chitosan with tripolyphosphate (TPP) than by spontaneous association. Dung et al. (2007) reported about chitosan-TPP nanoparticles as a release system for ASOs in the oral environment. The ability to control the characteristics of nanoparticles, most importantly the size and surface charge, is central in determining the efficacy of transfection (Nafee et al. 2007). Gao et al. (2005) demonstrated the use of galactosylated chitosan to deliver ASO efficiently to hepatic cells. Ozbas-Turan et al. (2009) investigated the effect of different formulation parameters [i.e., molecular weight and concentration of chitosan, concentration of tripolyphosphate (TPP), and use of alginate] on physicochemical and antisense properties of chitosan nanoparticles (NPs) encapsulating ASO which were designed to target the β-galactosidase (β-gal) gene. Efficiency of preparation methods of phosphodiester (PO)- and phosphorothioate (PS)-modified ASO nanoparticles was compared. In phosphorothioate ASO, a sulfur atom replaces oxygen at a non-bridging site of each phosphorous atom of chain. ASO-NPs were in general positively charged with size between 221 and 525 nm depending on formulations. PO-ASO encapsulation efficiency was less than PS-ASO encapsulation (91–98 %). The pH of chitosan solution affected encapsulation efficiency.

PO-ASO-NPs exhibited faster ASO release than NPs containing PS-ASO. In general, higher β-gal inhibition was obtained after transfection of ASO-NPs in HeLa cells. PS-ASO-NPs exhibited higher gene inhibition (90.71 %) than PO-ASO-NPs. Use of tripolyphosphate (TPP) as a cross-linker in nanoparticle preparation gave better result than use of alginate (Ozbas-Turan et al. 2009).

Chitosan was also used as a coating agent for liposomes (Wang et al. 2011) and PLGA nanospheres (Nafee et al. 2007; Tahara et al. 2008) for ASO delivery.

Intravenous injection of oligonucleotides has particular challenges (Wraight and White 2001). Skin delivery of ASOs provides a useful alternative to injection and has exciting potential in the treatment of skin diseases including skin carcinomas, psoriasis, inflammation, and herpes simplex (Wraight and White 2001). The skin is an attractive target for gene therapy because of its accessibility and immunologic functions which are important for vaccine delivery (Hengge et al. 1996). In addition to treating skin diseases, cutaneous ASO delivery can be used for achieving systemic effects. In spite of the advantages, dermal delivery of ASOs is still limited, and an appropriate nucleic acid carrier to overcome the skin barrier is needed. However, there are only few studies about topical application of ASO-loaded chitosan nanoparticles. At this stage, SMAD3 gene encodes a protein that belongs to the SMAD family (Smad3). In this study, ASO-impregnated chitosan-alginate polyelectrolyte complex was evaluated and applied for wound healing (Hong et al. 2008). They found that accelerated wound healing could be achieved by SMAD3 ASOs-PEC.

Dokka et al. (2005) investigated in mice skin dermal delivery via the follicular pathway of ASO formulated in a cream (containing glyceryl monostearate, hydroxypropyl, methylcellulose, isopropyl myristate, methylparaben, propylparaben, and polyoxyl-40-stearat). They investigated barrier function of epidermis and transport through skin and performed kinetic analysis of transport of ASO after application to mouse skin as lipophilic cream form. Their study showed that ASO penetration in skin involves a follicular route, and a vehicle/formulation with right hydrophilic/hydrophobic balance is required for effective transport of ASO within the skin (Dokka et al. 2005). Results from different studies reported gene suppression after topical administration of ASO, and different parameters controlled topical delivery of ASO (Mehta et al. 2000; Regnier et al. 1998; Wraight et al. 2000).

Ozbas-Turan et al. (2010) studied the ability of chitosan-tripolyphosphate (TPP) nanoparticles to deliver ASOs into the skin in adult and baby rats. Fifteen-nucleotide phosphorothioate oligonucleotides, targeting the sequence of β-galactosidase (β-gal) gene, were encapsulated into chitosan-TPP nanoparticles and topically applied to Sprague-Dawley rats (adult and baby). After transfection, at predetermined intervals, skin samples were taken for β-gal measurement. β-gal production was inhibited in approximately 82–85 % after nanoparticle application. ASODN-loaded chitosan nanoparticles showed long and sustained antisense effect in adult and baby rats. Similar results were obtained with skin DNA delivery. Skin samples of rat exhibit β-gal expression not only in epidermis but also in the dermis, particularly close to hair follicles (Ozbas-Turan and Akbuga 2011).

23.3 Chitosan/siRNA Nanoparticles

RNA interference (RNAi) is an endogenous regulatory mechanism of gene expression via small interfering RNA (siRNA) or micro RNA (miRNA) to promote messenger RNA degradation in a highly sequence-specific manner (Fire et al. 1998; Lee et al. 2012). Among the RNAi agents, synthetic siRNAs have been extensively studied as potential therapeutic molecules to overcome genetic disorders and other serious diseases such as cancer and viral infections. In 2001, Elbashir et al. (2001) reported that chemically synthesized 21-base pair double-stranded ribonucleic acid dsRNA molecules suppressed the genes with high specificity. Since then, significant progress has been made in siRNA delivery. Such

RNAi-based therapeutics including siRNA was clinically investigated for the treatment of age-related macular degradation, diabetic macular edema, respiratory syncytial virus infection, and cancers (Zhou et al. 2013). The siRNAs consist of 19–23 double-stranded ribonucleotides with two nucleotides overhanging at the 3′ ends and phosphates groups at the 5′ end (Elbashir et al. 2001; Martimprey et al. 2009; Zamore et al. 2000). In the cytoplasm, siRNAs are bound to a protein complex called the RNA-induced silencing complex (RISC) which contains the "silencing" protein Argonaute-2 that cleaves the target mRNA molecules. In general, the size of siRNA needs to be less than 22 base pairs to be used in the treatment of diseases. Longer siRNAs activate an interferon response, leading to the off-target effect by nonspecific mRNA degradation, and cause general inhibition of protein translation rather than the desired specific silencing (Elbashir et al. 2001). However, recently, some studies have shown that shorter siRNAs can induce nonspecific effect (Lee and Kumar 2009).

Two approaches use RNAi to inhibit target genes: siRNA and shRNA (short hairpin RNA). ShRNA is usually promoter dependent and can be delivered by plasmid or viral system. ShRNA is a DNA-based system in which the siRNA effectors are produced by intracellular processing of longer RNA hairpin transcripts. It is transported to the cytoplasm via the miRNA machinery and is processed into siRNAs by Dicer (Aagard and Rossi 2007).

Despite siRNA therapeutics are attractive molecules for human therapy, there are still remaining several challenges, such as the stability of molecules, off-target effects, and immune response (Bumcrot et al. 2006). These problems can be classified in two major issues: inherent problems of siRNA (i.e., stability in plasma, specificity, pharmacokinetic problems) and development of an efficient delivery system (Lee et al. 2012). Although chemical modification strategies provide high stability and reducing immunogenicity of siRNA therapeutics itself, a safe and efficient siRNA medicine is still needed as a suitable delivery system. Transportation of siRNA across the cellular membrane is hindered due to the hydrophilicity and negative charge of siRNA. Clinical application of siRNA is also restricted by its poor intracellular uptake and low target specificity when administered systemically (Lee et al. 2012). Chemical modification of the backbone was used to improve the stability and change the biodistribution of siRNA. Phosphorothioate-modified oligonucleotides induce high cellular uptake and improve blood half-life and biodistribution (Braasch et al. 2004), while incorporation of locked nucleic acid (LNA) molecules has been shown to increase serum stability and reduce off-target effect (Elmen et al. 2005). Other chemical modifications of siRNA include conjugation with polymers, peptides, and inorganic materials for improving specificity and targeting. For example, cholesterol conjugation to siRNA increased serum protein binding, altered biodistribution, and reduced urinary excretion (Soutschek et al. 2004). Naked siRNA showed rapid renal clearance ($t_{50} < 5$ min), while it could be extended to >30 min by cholesterol conjugation (Gao et al. 2009). Gao et al. (2009) have compared chemical modification of siRNA, nanoparticles such as chitosan nanoparticles, jet polyethyleneimine (PEI), and pegylated liposomal forms as strategies to improve systemic delivery of siRNA. Chemically modified siRNA could be detected in all organs 30 min after application but disappeared at 24 h except LNA-modified and cholesterol-conjugated siRNA in lungs. Nanoparticle formulations (chitosan, jet-PEI, and liposome) improved the stability and biodistribution of DNA; this is another strategy to improve the pharmacokinetic properties of siRNA (Gao et al. 2009). There have been several excellent reviews on siRNA delivery and its modifications (Lee et al. 2012; Martimprey et al. 2009; Aagard and Rossi 2007; Mao et al. 2010; Guo et al. 2010; Howard 2009; Urakami and Oku 2007).

In order to improve the therapeutic potential of siRNA, polycationic nanoparticles are used as carriers for siRNA. Because of its advantages, chitosan can be used as a carrier for siRNA.

The concepts and methods used in plasmid DNA delivery with chitosan can be adapted to siRNA.

23.3.1 siRNA Delivery

Compared to DNA delivery, very few studies are available about the chitosan-based siRNA delivery.

Howard et al. (2006) were the first developing chitosan/siRNA (chitosan mol wt 111 kDa, 84 % DD) nanocomplexes and studied the effect of N/P ratio on nanocomplex properties, such as size and zeta potential. Nanocomplexes in the size range of 40–600 nm were obtained. Size and zeta potential of nanocomplexes change with N/P ratio, i.e., the higher the N/P ratio, the smaller the particle size. All the chitosan/siRNA nanocomplexes have positive charge higher than 19 mV. In *Mus musculus* embryonic fibroblast cell line (NIH3T3) cells, rapid cellular uptake was observed of cyanine-5 (Cy-5)-labeled nanocomplexes. Higher endogenous EGFP inhibition was demonstrated in human lung carcinoma cells (77.9 % reduction) and murine peritoneal macrophages (89.3 % reduction in EGFP).

The same group investigated also the effect of chitosan properties [molecular weight and degree of deacetylation (DD)] and N/P ratio on physiochemical properties and silencing efficiency of chitosan/siRNA nanoparticles (Liu et al. 2007). According to their data, physicochemical properties such as zeta potential, size, and complex stability of the chitosan/siRNA nanoparticles were indicated to be dependent on the molecular weight and DD of chitosan. The most stable chitosan/siRNA nanoparticles were formed at N/P ratio 150 and using high molecular weight (114 and 170 kDa) and high DD (84 %) chitosan. These NPs exhibited high (~80 %) *in vitro* gene knockdown in *Homo sapiens* lung carcinoma cell line (H1299) green cells which was comparable to TransIT-TKO® (Mirus Bio LLC), being a commercial vector.

Katas and Alpar (2006) studied three methods of siRNA association to chitosan/TPP nanoparticles.

For clinical use, obstacles such as instability, renal clearance, and distribution of naked siRNA are important drawbacks. Chemical structure of siRNA backbone has been modified for improving the stability and altering the biodistribution of siRNA.

Salva et al. (2011) investigated the biodistribution and tumor localization of chitosan/vascular endothelial growth factor (VEGF)-siRNA complexes in breast cancer model of rat. While the biodistribution of complexes to the brain and heart appeared similar, higher accumulation in the kidney was observed. This result related to kidney accumulation shows similarity with Guo's data (Guo et al. 2011). Various salt forms of chitosan including chitosan acetate, chitosan aspartate, chitosan glutamate, and chitosan hydrochloride formed stable complexes with siRNA.

shRNA-targeting vascular endothelial growth factor A was complexed with chitosan (mol wt 75 kDa and 85 % of DD), and its gene silencing capacity was investigated in 4 different cell lines, two of which are MCF-7 and MDA-MB-435 breast cancer cell lines. Sixty percent of VEGF inhibition was measured in MCF-7 cells after transfection, while the lowest (29 %) inhibition was found in MDA-MB-435 cell line. Nanoplexes indicated cell-type-dependent transfection efficiency. These differences may be due to numbers of VEGF receptors present on the cell surface as previously mentioned by Tan et al. (2007) and Salva et al. (2010).

Chitosan and trimethylated chitosan siRNA complexes indicate similar characteristics for siRNA delivery (Dehousse et al. 2010).

Non-arginine-modified chitosan (Park et al. 2013), chitosan-oligoarginine (Park et al. 2011), and chitosan-poly-L-arginine-PEG (Noh et al. 2010) were evaluated as delivery systems for siRNA.

23.4 Intracellular Trafficking of Chitosan-Based Delivery Systems

To improve and optimize the transfection ability of chitosan-based delivery systems, it is important to understand the intracellular trafficking of this DNA carrier and to identify specific rate-limiting steps to gene expression (Thibault et al. 2010). Intracellular DNA unpacking and release from carrier are regarded one important rate-limiting

system. On the other hand, generally therapeutics of molecular weight larger than 1 kDa does not cross the cellular membrane, and cellular uptake is limited to the endocytic route. After nanoparticles interact with exterior of membrane, they can enter the cell through a process called "endocytosis" (Sahay et al. 2010). Endocytosis involves multiple stages: endosomes (phagosomes) and trancytosis. Different endocytic pathways such as clathrin-dependent endocytosis (CME) and clathrin-independent endocytosis (Conner and Schmid 2003) are present. The clathrin-independent pathways are classified as caveolae-mediated, clathrin, and caveolae-independent endocytosis and macropinocytosis. Although endocytosis is common to all cell types, certain pathways are cell-specific or display more expression in certain cells. A nanomedicine may enter cells via a number of different pathways at the same time. Internalization efficiency may depend on the number of binding site receptors per cell, the rate of receptor recycling, and the effect of the cargo on receptor up-/downregulation (Duncan and Richardson 2012). Macromolecules can be inactivated by acidic pH and/or rapid hydrolysis by lysosomal enzymes. Therefore, it is needed to protect the nucleic acids during their transit through the cytosol and promote their efficient delivery to the desired intracellular compartment (nucleus). Between the therapeutic nucleic acids, synthetic siRNA molecules are >100-fold smaller than a gene, and the final destination in the cell is cytosol rather than nucleus (Duncan and Richardson 2012). Endosome-tropic delivery is more challenging. Carrier triggered by lowering the pH of early endosomes after its internalization is able to transiently permeabilize the early endosome-late endosome membrane allowing a cargo to enter the cytosol. In this subject, very useful reviews are present in literature (Sahay et al. 2010).

Thibault et al. (2010) investigated intracellular trafficking of chitosan-based polyplexes to identify processing events that are sensitive to molecular weight and deacetylation degree. They showed that the kinetics of polyplex decondensation is the most critical formulation-dependent intracellular process. The most efficient chitosans indicated an intermediate stability and a dissociation kinetic which occurred in synchrony with lysosomal escape. This was found to be dependent on chitosan structure. They reported that their findings involving lysosomal sequestration are in contradiction with earlier studies suggesting that chitosan polyplexes do not transit through lysosomes at all or only for short periods (within 4 h) (Huang et al. 2005; Kiang et al. 2004; Chen et al. 2008). According to Thibault et al. (2010), this contradiction can be due to different cell types, different chitosans, and use of different methodologies for transfection and lysosomal staining. Nuclear entry of plasmid DNA is another important barrier in intracellular trafficking. According to Thibault et al. (2010), nuclear uptake of pDNA is not facilitated by chitosan, but is a function of the availability of cytosolic pDNA. Once the plasmid DNA is released to the cytosol, the SV40 enhancer sequence of the plasmid increases nuclear translocation of plasmid in the absence of cell division. Large nanoparticles have been taken up by macropinocytosis and clathrin-mediated pathway, whereas smaller nanoparticles are internalized through caveolin-mediated endocytosis (Grosse et al. 2005). However, the reports on size dependency are not consistent. Rejman et al. (2004) suggested that larger particles (>500 nm) are taken up by caveolae-mediated pathway, while smaller particles (<200 nm) internalized by clathrin-mediated endocytosis.

23.5 Stability of Chitosan/DNA Nanoparticles

A good gene delivery system requires besides efficiency also stability during storage. All nanomedicines should be evaluated for their thermal stability under storage conditions (Muthu and Feng 2009). Although many papers have been published on strategies to improve the transfection efficiency of this system, few papers have been published yet on the stability of these systems (Leong et al. 1998; Romoren et al. 2004).

Romoren et al. (2004) reported that the biological and physicochemical stability of chitosan-

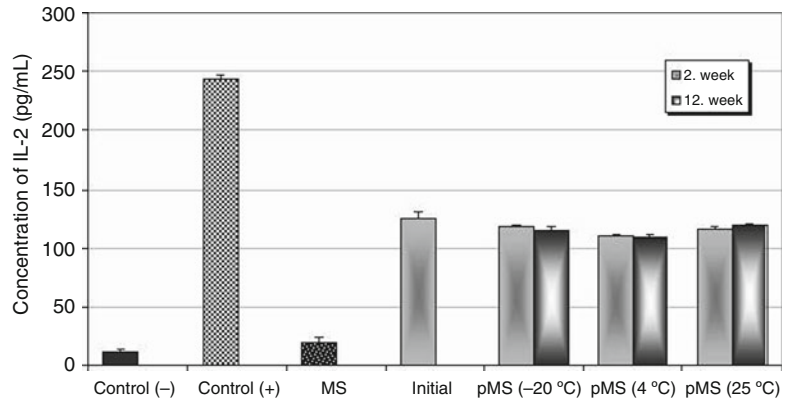

Fig. 23.1 *In vitro* stability of pIL-2-loaded chitosan microspheres stored at different temperatures (−20, 4, and 25 °C)

based polyplexes is maintained for 1 year during storage in acetate buffer at 4 °C. Leong et al. (1998) showed that chitosan/DNA nanoparticles stored in water remained stable for more than 3 months, whereas uncross-linked nanoparticles stored in PBS remained stable for a few hours only. Akbuga et al. (2002) showed that stability and *in vivo* transfectional potential of encapsulated pDNA did not change during storage at −20, 4 and 37 °C for 3 months. Ozbas-Turan et al. (2002) investigated the stability and transfection efficiency of pIL-2-loaded chitosan microspheres after storage at different temperatures (4, 25 and −20 °C). It was observed that the size of particles was slightly increased when they were stored at 25 and −20 °C. *In vitro* pDNA release from microspheres slightly changed depending on storage conditions. However, IL-2 production was found to be quite similar after 2 and 12 weeks storage at different temperature. As a conclusion, pIL-2 was protected well in chitosan microspheres (Fig. 23.1).

Conclusions

In conclusion, antisense oligonucleotides and RNAi-based therapeutics are a powerful new approach for treatment of skin diseases. However, there are still some challenges such as off-target effects, delivery to target cells, and stimulating immune responses, to be overcome in order to be used as therapy. The key problem for the application of these molecules is the use of an appropriate delivery system. Chitosan and modified forms are useful and effective delivery systems for ASO- and RNAi-based therapeutics. ASOs as cutaneous administration with chitosan-based systems provide advantages over intravenous ASOs. Skin delivery will provide direct access to target cells in the skin and will decrease systemic toxicity. On the other hand, very little information is available for siRNA skin delivery. However, more studies are needed to be performed before the clinical application of chitosan-based forms of carriers.

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Safety of Nanoparticle Skin Penetration

24

Nancy A. Monteiro-Riviere

Contents

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24.1 Introduction

Exposure to nanomaterials in many occupational, consumer, cosmetic, and environmental scenarios often occurs primarily to the skin. A relatively large number of studies have been conducted in these areas, although differences in the nature of materials studied, their degree of characterization (size, shape, surface coatings, vehicles/formulations), as well as the quantity and mode of material exposure make obtaining a fundamental understanding of nanomaterial skin safety elusive. There remains a paucity of safety and toxicology data conducted over longer exposure periods (days to months) that characterizes many patterns of routine usage.

The skin is fundamentally different from other routes of exposure in that nanoparticles are directly deposited onto the skin's surface without having to first pass through an environment that could alter their physiochemical properties. When nanoparticles are ingested, they first encounter saliva and other digestive fluids (gastric and intestinal juices) before reaching the absorptive surface of the gastrointestinal mucosa. Chemical processes in the digestive system have been shown to alter silver nanomaterial properties (Liu et al. 2013). When inhaled, aerosolized particles traverse the complex bronchiolar pathways, and depending on size are either deposited onto mucous covering the bronchiolar epithelium or onto surfactant-containing fluid lining the

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alveoli. In both the oral and inhalational scenarios, deposited particles dissolved or suspended in a solution will contact living cells, many of which possess phagocytic and other particle transport processes related to physiological scavenging functions. This is contrasted to the exterior surface of the skin which is inert and consists primarily of the acellular stratum corneum, a water impermeable barrier. In most mammalian species, the stratum corneum is perforated by hair shafts and follicles as well as sweat gland ducts, all of which vary in densities as a function of species and body region. It is only after a topically applied nanomaterial traverses either the stratum corneum or the lining of the hair follicles could they encounter viable cells and potentially elicit a toxicological response. Resident epidermal dendritic cells with processes just below the stratum corneum barrier may elicit an immune response after ingestion of only a few nanoparticles. Further penetration could potentially result in delivery to the capillary beds or lymphatics which could result in systemic exposure or interact with other cellular members of the immune system that could also elicit systemic or local effects.

Topical skin exposure is very different from internal routes of exposure because its surface is affected by ambient conditions (temperature, humidity, UV sunlight exposure, sweat, compromised or diseased skin, presence or absence of clothing) that can affect both particle-particle and particle-skin interactions. In the majority of topical exposure scenarios, nanoparticles will be carried in some type of formulation (cosmetic, dermatologic) or vehicle (occupational, environmental) that may affect the state of individual nanoparticle aggregation. Often pristine nanoparticles could be deposited onto the skin and be unable to directly interact with the stratum corneum unless they became solvated by sweat or exogenous liquid sources. For some specific topical skin applications of nanomedicine, particle-formulation pairs may be specifically optimized either to maintain activity of the skin surface without dermal penetration (e.g., sunscreens, surface antimicrobials), to penetrate hair shafts for sustained delivery to this region (e.g., antifungals etc.), or alternatively be formulated to avoid aggregation and have surface chemistries compatible with partitioning into the intercellular lipids of the stratum corneum: the pathway for dermal absorption to deeper skin layers or the capillary beds and systemic delivery.

24.2 What Are the Biological Targets Within the Skin and How Does the Skin Respond to a Toxic Insult?

There are many comprehensive reviews on the skin which have been reviewed elsewhere (Monteiro-Riviere 1991, 2006, 2008, 2010; Monteiro-Riviere and Baroli 2010). The skin is the largest organ in the body and is the body's interface with the environment. It is primarily an organ responsible for protecting the body from external insults that are manifested by its barrier, metabolic, ultraviolet (UV) light shielding, and immunological functions, which respectively reside in the stratum corneum, metabolically active basal epidermal keratinocytes, UV-absorbing melanocytes, and the immune system components residing within the skin (keratinocytes, resident mast cells, and dendritic Langerhans cells). Another important function of mammalian skin is thermoregulation mediated by insulating fur/hair, heat-dissipating sweat, and a high density of heat-dissipating blood capillaries that far exceed metabolic demands. The skin is an important outpost of the nervous system involved in sensing pressure and heat and releasing neural-active mediators to produce pain in the affected areas. It is an endocrine organ involved in synthesis of vitamin D, androgens, estrogens, and insulin-like growth factor-binding protein-3, as well as serving as a target of numerous systemic hormones (Monteiro-Riviere 2010; Zouboulis 2009). Finally, the skin plays a major role in social interactions by determining the appearance of the species and individual to others, a function mediated by fur/hair, skin color determined by melanocyte density, skin texture controlled by the integrity and elasticity of the supporting subepidermal structure, efficacy of the stratum corneum barriers to produce a soft

and pliable surface, and sexual attraction mediated by pheromone excretion.

All of these attributes must be considered when assessing the nature of nanoparticle-induced skin toxicity, since abnormalities in these functions determine the nature of the dermal response to a toxic insult. All of these skin functions may only be modulated if the nanomaterial or small molecule physically reaches and interacts with living tissue. In contrast to smaller organic chemicals and drugs which may partition into the intercellular lipids that form the stratum corneum barrier, nanomaterials are primarily restricted by their large sizes which limit most nanomaterials from penetrating into the viable epidermis, dermis, or being absorbed into the systemic circulation. If nanoparticles can penetrate to and interact with skin elements, then the responses seen are characterized by the degree of modulation of normal skin functions. Nanomaterial-induced irritation, characterized by the skin's reaction to what it senses as a noxious threat, is the simplest toxicological reaction that can be initiated by nanomaterial exposure. This is known as direct irritation and may be defined as an adverse effect of compounds directly applied topically to the skin not involving prior sensitization and thus initiation by an immune mechanism. Several types of adverse effects and chemical interactions may be manifested when a nanomaterial comes in contact with the skin surface. Irritation is usually assessed by local cardinal signs of inflammation characterized by erythema (redness), edema (swelling), heat, and pain. Other responses may be present that do not elicit inflammation such as an increase in thickness. Irritant reactions may be classified by many characteristics including acute, cumulative, traumatic, and pustular; however, two classifications are generally studied by toxicologists. Acute irritation is a local response of the skin usually caused by a single agent or nanomaterials which induces a reversible inflammatory response. Cumulative irritation occurs after repeated exposures to the same irritant and is the most common type of irritant dermatitis seen. The mechanisms of irritation are thus very complex and are still being characterized since they involve interaction of inflammatory cytokines with subsequent involvement with the immune system. This dermal irritation response is mediated by a complex cytokine cascade initiated by a wide variety of insults, be it chemicals, microbial invasion, contact allergens, physical trauma, nanomaterials/nanoparticles, or exposure to UV light. The cytokine cascade relative to skin structure is very complex.

When epidermal cells are affected, they may initiate other sequelae. If a compound or nanomaterial is capable of penetrating the skin and interacting with the immune system, the manifestations seen will be dependent upon the type of immunologic response elicited (e.g., cellular versus humoral, acute hypersensitivity, etc.). It should be noted that immune cells such as Langerhans cells, lymphocytes, and mast cells may modulate a reaction, or the keratinocytes themselves may initiate the response. In fact, keratinocytes were once thought to produce only keratin and mucopolysaccharides, but now it is known that they can produce growth factors, chemotactic factors, and adhesion molecules. Keratinocytes may also act as the key immunocyte in the pathophysiology of allergic contact and irritant contact dermatitis. Skin contact with irritants may trigger the production of proinflammatory cytokines. Direct irritation of keratinocytes by toxic chemicals or nanomaterials may also initiate a cytokine cascade without involvement of the immune system, blurring the distinction between direct and indirect cutaneous irritants. The interested reader should consult dermal immunology texts (Nickoloff 1993) for further details on the broad range of immunologic manifestations possible.

The primary mechanism of many topical irritants such as organic solvents or corrosives is the impairment to the stratum corneum barrier properties discussed earlier, reflected by an increase in transepidermal water loss (TEWL). If the stratum corneum barrier is perturbed, a feedback response may be initiated whereby regeneration of the barrier occurs. This reaction is mediated by cytokines especially tumor necrosis factor alpha (TNF-α) originating locally within the epidermis. However, additional responses

to these inflammatory mediators may in themselves launch an irritation response mediated by the keratinocytes. Relative to manufactured/engineered nanomaterials and topically applied nanomedicines, this response could be theoretically initiated by:

- Activation of the skin allergic response via nanoparticle interaction with dendritic cells
- Nanoparticle alterations in barrier function through penetration and disruption of the stratum corneum layers
- Direct interaction with epidermal keratinocytes resulting in irritation and cytokine release or direct toxicity resulting in cell death and subsequent irritation

It must be stressed that nanomaterials do not need to fully penetrate the epidermis and interact directly with keratinocytes nor do they need to be absorbed into the system circulation to initiate dermal irritation or an immune or sensitization response. Disruption of barrier function alone by penetration into or interaction with stratum corneum lipids could produce a perturbation that results in dermal irritation (Elias 2006; Nickoloff 1993). Additionally, nanoparticles only need to penetrate into the deeper layers of the stratum corneum to reach the dendritic processes of Langerhans cells to initiate an immune response. This is the target for delivering antigens using topical vaccination strategies.

The second type of potential nanomaterial-induced dermatotoxicity involves interactions that modulate epidermal and dermal cellular functions without causing massive cell death or cytokine release. These include inducing proliferation or neoplastic transformation of keratinocytes or melanocytes, resulting in subtle changes and a chronic rather than acute response. The ability of nanomaterials to induce these effects has not been extensively studied. Most in vivo studies with nanomaterials or nanoparticles have been short term and only assessed loss of barrier functions by TEWL or as sensitization, inflammation, and proliferative responses. In vitro experimental endpoints are geared toward irritation (cytokine release) and direct keratinocyte toxicity (loss of viability, visualization of cell death, or markers of oxidative damage, etc.). The present state of nanomaterial dermatotoxicology is limited to assessment of acute and short-term responses, primarily using in vitro model systems.

One of the major impacts on experimentally assessing dermatotoxicity is the animal species employed since differences in anatomy and physiology modulate the response potentially seen. The most obvious variables are differences in hair density and skin thickness that can modulate particle penetration. Since most laboratory rodents used in experimental studies have a dense fur coat, the question often arises of their applicability to predict responses in humans whom do not have a dense fur coat or very thin skin. Even within species, there are regional differences in hair density and skin thickness that may modulate responses (Monteiro-Riviere et al. 1990; Monteiro-Riviere 2006, 2008). Different species have different immunological systems, not all triggered by skin interactions. Finally, the molecular organization and biochemical composition of stratum corneum lipids are different between species and could affect results obtained (Monteiro-Riviere et al. 2001). The comparative biology of the skin across different species has been extensively studied (Monteiro-Riviere 1991, 2006, 2010; Monteiro-Riviere et al. 1990) and should be consulted by investigators first before nanomaterial studies are conducted to insure that responses seen in experimental studies are capable of occurring in humans.

24.3 What Is the Current Paradigm for Assessing the Cutaneous Safety of Nanomaterial Dermatotoxicity?

Based upon the above considerations, what is the current paradigm for assessing cutaneous safety of nanomaterials? There are three very distinct avenues of investigation utilized: assess toxicity and irritation with in vitro epidermal keratinocyte cultures, assess ability of nanoparticles to pene-

trate skin using dermatomed skin mounted in static or flow-through in vitro diffusion cells, or conduct short-term (hours to few days) in vivo topical exposure studies.

24.3.1 In Vitro Cell Culture

Due to the rapid advancement in nanotechnology, the safety of nanomaterials is of concern. There are numerous nanomaterial dermal toxicity assessments conducted using in vitro human epidermal keratinocytes (HEK) in culture (Monteiro-Riviere et al. 2005, 2009, 2010; Monteiro-Riviere and Inman 2006; Rouse et al. 2006; Ryman-Rasmussen et al. 2007; Samberg et al. 2010; Saathoff et al. 2011; Zhang and Monteiro-Riviere 2009a; Zhang et al. 2007, 2008). It is well known that many in vitro studies are used to predict a human response. Standard protocols used in assessing chemical or drug toxicity have been employed for nanomaterial/nanoparticle toxicity. Response is also assessed morphologically by light or electron microscopy or by standard classical in vitro cytotoxicity assays in cultured cells with calcein AM, Live/Dead® (LD; Invitrogen, Carlsbad, CA, neutral red (3-amino-7-dimethylamino-2 methylphenazine hydrochloride), MTT (3-[4,5-dimethyl-2-thiazol]-2,5-diphenyl-2H-tetrazolium bromide), CellTiter 96® AQueous One (96AQ ((CTB; Promega Corp, Madison, WI), alamarBlue, CellTiter-Blue®, CytoTox-ONETM Promega Corp, Madison, WI), homogeneous membrane integrity, trypan blue, and flow cytometry (Monteiro-Riviere et al. 2009) or cytokine release (interleukin (IL)-8, in addition to IL-6, IL-10, IL-1β, and TNF-α. The expression of IL-8 release in keratinocytes plays an important role with nanoparticle exposure. The classical dye-based assays mentioned above that determine cell viability may produce invalid results with some nanomaterials due to nanomaterial/dye interactions and/or nanomaterial adsorption of the dye/dye products. These assays provide accurate viability data for classic small molecule toxicity studies but have proven to be less reliable when assessing nanomaterial or nanoparticle toxicity (Monteiro-Riviere and Inman 2006; Monteiro-Riviere et al. 2009). Results showed that the optimal high-throughput assays for carbon and noncarbon nanomaterials were 96AQ. Also, the results of these studies showed that unlike small molecules, carbon nanomaterials can interact with assay markers to cause variable results with these classical toxicology assays. Our studies showed that viability/cytotoxicity assays alone may not be adequate at this time to evaluate nanomaterial effects in cell culture due to the nanoparticle agglomeration and the interactions of the nanomaterial with dyes and dye products. It is extremely important to conduct ultraviolet/visible absorbance (UV/Vis) on the nanoparticle in the assay medium to see if there is a shift in absorbance or amplitude in the no-cell controls. Multiple assays should be employed depending on the type of nanomaterial in addition to imaging techniques such as transmission electron microscopy to validate chemical marker-based viability assays. These different cytotoxicity results reported with nanomaterials will make health assessments difficult and more perplexing to interpret when conducting health risk studies of nanomaterials. Many nanomaterials may or may not have a potential toxic effect; inaccurate screening could hinder the progress of nanotechnology due to erroneous results.

Specific examples of where these assays have been employed are discussed in the specific nanomaterial examples below. Additional studies have been conducted to determine the mechanism of HEK cellular uptake to define potential pathways. An excellent example of the different endocytic pathways that were studied depicts nanoparticle cellular uptake of quantum dots (QDs) into HEK (Zhang and Monteiro-Riviere 2009b). QD nanoparticles were selected due to their intense photostable fluorescent properties and availability in various sizes, shapes, and surface coatings. This was one of the first studies that showed that QD nanoparticles with a carboxylic acid surface coating were recognized by lipid rafts and not by clathrin or caveolae in HEK. Also, it was demonstrated that QDs were internalized into early endosomes and then transferred to late endosomes or lysosomes. Utilizing twenty-four

endocytic interfering agents to investigate the mechanism, by which QD entered cells, depicted that QD endocytic pathways were primarily regulated by the G protein-coupled receptor associated pathway and low density lipoprotein receptor/scavenger receptor. Other studies were conducted in our laboratory to determine if lectins modulate multiwalled carbon nanotube uptake in HEK. Since keratinocytes normally engulf melanosomes, we hypothesized that the melanocyte transfer pathway could serve as a potential route of entry into HEK. Lectins are inhibitors of the melanosome transfer pathway, and three different lectins were used to study the uptake of multiwalled carbon nanotubes in HEK, to see if they played a role in reducing the cellular uptake of multiwalled carbon nanotubes. A lectin cocktail of three lectins showed a decrease in multiwalled carbon nanotube interaction at the cell surface and uptake (Zhang and Monteiro-Riviere 2010).

24.3.2 Limitations of In Vitro Assays Designed for Chemicals Applied to Nanomaterial Safety Assessment

There are significant limitations of in vitro cell culture systems, designed for small soluble molecules, applied to colloidal nanoparticle exposures (Monteiro-Riviere and Inman 2006; Monteiro-Riviere et al. 2009). The major limitation is that certain nanomaterials directly interact with classic cell viability biomarkers and cause both false-positive and false-negative results. One of the hallmark properties of nanoparticles that makes them unique for many applications is their extremely large surface area to mass ratios which provides enhanced surface-adsorptive properties and provides a large sink for nonspecific adsorption of molecules, including cell toxicity assay products and nutrients essential for cell viability under culture conditions.

The in vitro cell culture-based cytotoxicity screening assays are designed to minimize animal use in toxicity testing and have become the mainstay of modern toxicology testing strategies (National Research Council 2007). It is not always feasible to use animals in testing in the United States or European Union. Since the EU will not allow any in vivo testing in animals for cosmetics, in vitro testing becomes even more important requiring some assurance that these results reflect in vivo behavior. While many of these new nanomaterials may or may not have a potential toxic effect, inaccurate screening could impede nanotechnology progress due to erroneous results.

An additional concern is more theoretical and relates to how in vitro studies may be extrapolated to the in vivo setting. Under normal in vitro cell culture conditions, cells are bathed in a nutrient-rich protein-containing aqueous medium. For keratinocytes, this may not reflect their in vivo environment in the skin where viable keratinocytes are sandwiched between the basement membrane and nonviable stratum corneum and not in direct contact with protein-rich fluids. Two properties of nanomaterials, which are very different from chemicals and drugs, are their propensity to aggregate (nanoparticle to nanoparticle interaction) and/or agglomerate (nanoparticle-protein-nanoparticle interaction) under the appropriate conditions within the cells. Aggregation is sensitive to the properties of the solution in which a nanoparticle is solvated. Differences in hydrophobicity, salt concentration, and pH will affect this phenomenon. Agglomeration, and more specifically nanoparticle-protein corona formation, will also affect the physical chemical properties of the nanoparticle's presentation to cellular receptors, that is, how the cell "sees" the nanoparticle in that a nanoparticle may form specific protein coronas with the protein constituents of cell culture medium (Lynch et al. 2007; Shannahan et al. 2013). Recently, our laboratory has shown the importance of these interactions to HEK uptake of a variety of silver nanoparticles. Preincubation with corona-forming proteins changed the extent and nature of HEK uptake compared to exposure to native uncomplexed particles (Monteiro-Riviere et al. 2013). This finding raises the question of how relevant in vitro cell culture studies using aqueous protein media is to HEK uptake of

nanoparticles in vivo where corona formation may not be anticipated.

Figure 24.1 is a transmission electron micrograph that depicts aggregates of 60 nm silicon dioxide-coated Fe_2O_3 in HEK for 24 h. These interactions, which may both increase the size of the nanomaterial and mask nanoparticle surface chemistries, are more likely to occur in the aqueous conditions seen in cell culture medium rather than in the intercellular lipid domains of the stratum corneum layer through which nanoparticles must pass to reach the viable HEK in vivo. Because of this limitation, cell culture systems do not possess the stratum corneum rate-limiting barrier to topical nanomaterial penetration, which in some ways could be considered a filter to the types of nanomaterials that could reach HEK in vivo. Another limitation is the relationship between the concentrations in vitro that elicits a response and that which is seen in vivo under normal exposure conditions. Finally, dendritic cells in culture have been used to assess the nanoparticle cellular uptake into these immunologically critical cells (Zhang et al. 2011). This study showed dendritic cells that were differentiated from monocytes resulted in a sixfold increase in QD uptake.

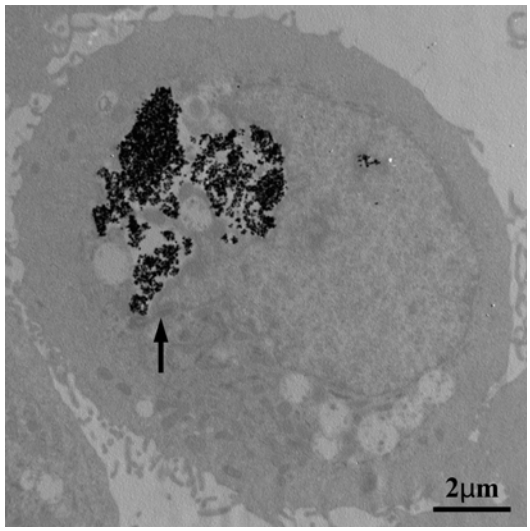

Fig. 24.1 TEM of HEK treated 60 nm silicon dioxide-coated Fe_2O_3 in HEK for 24 h. *Arrow* depicts aggregates of silicon dioxide-coated Fe_2O_3

24.3.3 In Vitro Skin Penetration Models

The next experimental approach directly addresses the assumption that for a nanomaterial to be toxic to the skin, it must first penetrate the stratum corneum. This line of reasoning assumes that if the particle cannot penetrate the stratum corneum barrier, it cannot be toxic to the viable skin. To test this hypothesis, standard in vitro diffusion cell models normally employed to assess chemical and drug dermal absorption have been used. These systems have been well described in the literature (Bronaugh and Maibach 1991). The primary differences between systems used in nanotoxicology studies are type of diffusion cell, method of membrane preparation, species from which skin membrane is harvested, composition of the receptor fluid, and method of topical nanomaterial application (neat, vehicle). The two primary types of diffusion cells used are static (e.g., Franz cells) (Frantz 1978) or flow-through (e.g., Bronaugh cells) (Bronaugh and Maibach 1991; Bronaugh 2006) systems that differ only in the nature of the perfusate bathing the dermal side of the skin, static cells have fixed media from which samples are taken, while flow-through cells perfuse media to mimic blood flow under the skin. Membranes could include full-thickness skin, skin dermatomed to specific thicknesses, or epidermal membrane heat separated from the underlying dermis. Some pure non-biological membranes, such as the parallel artificial membrane permeability assay (PAMPA) employed to study chemical and drug dermal absorption, may not be appropriate for nanomaterials (Sinko et al. 2012; Karadzovska and Riviere 2013) since nanomaterials may simply dissolve in these amorphous membranes and not face the size restrictions seen in intact stratum corneum cell layers.

Many animal species have been utilized, human skin obtained from surgical procedures (viable), or cadavers (dead) and pig skin predominating in studies designed to assess absorption in humans. Haired laboratory rodents with very thin skin are often employed due to their easy availability, but extrapolation to

humans may be problematic. A unique concern with nanoparticle is their binding to the surface of hairs in furry species. Shaving rather than clipping of hair may introduce nicks or small cuts in the skin that would allow for nanoparticle penetration. Another limitation of in vitro systems is differences in elastic modulus between the species. This is a concern when human skin is employed in vitro since it contracts when collected. The membrane should be stretched when mounted onto a diffusion cell. This is not seen with other species such as pigs, and its effect on extrapolating nanoparticle penetration data from in vitro to in vivo human penetration is not known, since contraction of the skin in vitro may increase the nanoparticle barrier compared to the in vivo state.

Endpoints of nanoparticle penetration include the fraction of dose detected in perfusate reflecting nanomaterial absorption or transdermal delivery, amount of dose found deposited in various skin fractions (stratum corneum tape strips, sectioned skin biopsies), or nonabsorbed amount left on the surface of the skin (surface wipes and washes). For fluorescent materials such as QD, confocal laser microscopy is often used to visualize QD penetration in skin sections obtained from diffusion cells after exposure (Zhang and Monteiro-Riviere 2013). More sophisticated quantitative imaging tools such as time-of-flight secondary ion mass spectrometry (TOF-SIMS) have been employed (Monteiro-Riviere et al. 2011b). Finally, biopsy sections may be examined by transmission or scanning electron microscopy (TEM, SEM) to specifically localize particles within cells or organelles.

Despite concern to the contrary, intact nanomaterial absorption into perfusate has not been reproducibly detected using all of the analytical methodologies commonly employed in any absorption study. The methods used to assess absorption are a function of the material composition of the nanomaterial being studied. Methods employed have included all varieties of gas and liquid chromatography, fluorescence and mass spectrometry, and inductively coupled plasma mass spectroscopy (ICP-MS). From the literature examined to date for all types of nanomaterials in all species including humans, significant flux of nanomaterials applied on the surface of the skin have not been detected in perfusate at the limit of sensitivity for the analytical method employed.

24.3.4 Solvent Effects

The nature of the solvent or vehicle in which the nanomaterial is applied to the skin's surface is important since most nanomaterials are exposed as solutions or colloid suspensions due to their particulate nature or for stability. The application vehicle or formulation may have profound effects on the amount of penetration seen. Our laboratory showed that when pristine hydrophobic fullerenes (C_{60}) were applied topically to in vivo porcine skin in a series of organic solvents (toluene, cyclohexane, chloroform) or mineral oil for four days, chloroform resulted in the deepest C_{60} stratum corneum penetration compared to the other solvents, while no penetration was detected when dosed in mineral oil (Xia et al. 2010a). Vehicle modulations (oil in water versus water in oil) were also seen in the depth of stratum corneum penetration of titanium dioxide applied as sunscreen formulations in porcine skin (Monteiro-Riviere et al. 2011b). Across all particles, such vehicle effects may be modulated by vehicle/formulation effects on solubility, nanoparticle aggregation state, effects of a vehicle's ionic strength and pH of particle surface charge, and vehicle interactions with the stratum corneum lipid barrier (solvents, high pH). Additional studies with terpenes were also evaluated to determine if terpene exposure would modulate topical nanoparticle dermal penetration by pretreating human skin for 1 h with terpenes such as menthol, eucalyptol, limonene, or ethanol vehicle (Monteiro-Riviere et al. 2011a). Since terpenes are highly lipophilic aliphatic compounds and are known to alter the stratum corneum lipids, it was hypothesized they could alter the barrier properties to allow for an increase in penetration. In vitro flow-through diffusion cell studies with fresh human skin were conducted for 8 h and dosed with two different sizes of QD. Laser scanning confocal microscopy of the

human skin showed no QD penetration into the viable layers, but detected QD in layers of the upper stratum corneum. TEM showed QD penetration slightly deeper into the stratum corneum in terpene pretreated skin, yet QD were not detected in the perfusate. These studies concluded that terpenes caused only a slight enhancement of QD penetration into the stratum corneum layers, but no transdermal flux occurred across the skin (Monteiro-Riviere et al. 2011a).

There is another characteristic of nanoparticle interactions with the stratum corneum barrier lipid pathways that is fundamentally different than what is seen with most chemicals and small molecules: the potential for a particle partitioned into the stratum corneum to subsequently cause barrier disruption and further penetration (Cevc and Vierl 2010; Monteiro-Riviere and Baroli 2010). The stratum corneum is not a spatially uniform structure but instead can be viewed as vertical columns of flattened keratinocytes, with uniform intercellular spaces filled with organized lipids within the columns, but less uniform spaces between adjacent columns and clusters of columns and where adnexal structures such as hair or sweat ducts ascend up to the skin's surface. Intercellular spaces in these peripheral "edge" regions may be as wide as 20–30 nm, well within the size range to allow penetration of many topically applied nanoparticles. In fact, some nanoparticles have been reported to preferentially locate in this region (Baroli et al. 2007; Zhang et al. 2008). These border areas may be associated with relatively hydrophilic transepidermal aqueous pathways that could be capable of generating a transepidermal water gradient under the proper conditions that would facilitate transport of small water-soluble nanomaterials. Once a nanomaterial, with a reactive surface, penetrates the intercellular spaces, disruption of the lipid barrier could also occur thereby, facilitating penetration.

24.3.5 Mechanical Actions

Despite a number of potential mechanisms and pathways for nanomaterials to traverse the skin barrier, to date nanoparticle penetration into the skin has been detected, but not substantial transdermal flux across the epidermis. Some research has demonstrated that mechanical action and perturbations of the skin barrier can affect the penetration of nanoparticles. Tinkle et al. (2003) reported that large 0.5 μm fluorescein isothiocyanate (FITC)-conjugated dextran beads could penetrate the stratum corneum of human skin and reach the epidermis following 30 min of flexing. Normally, these particles would not penetrate the skin. Very small bucky amino acid (Baa) fullerene-substituted phenylalanine derivative of a nuclear localization peptide sequence (Baa-Lys(FITC)-NLS)-derivatized fullerene nanoparticles of 3.5 nm were able to penetrate into the dermis of porcine skin that was flexed for 60 min and placed in flow-through diffusion cells for 8 h. The nonflexed control skin showed penetration limited to the stratum granulosum layer of the epidermis (Rouse et al. 2007). Other studies were conducted in our laboratory with QD655 and QD565 coated with carboxylic acid (hydrodynamic diameters of 18 and 14 nm, respectively) in flow-through diffusion cells with flexed, tape stripped, and abraded rat skin for 8 and 24 h. Penetration was not observed with the nonflexed, flexed, or tape-stripped skin, but penetration to the viable dermal layer occurred in the abraded skin. In some cases, retention of the QD in hair follicles was noted in the flexed skin (Zhang and Monteiro-Riviere 2008).

Another factor that has been postulated to increase dermal nanomaterial absorption is the presence of damaged or diseased skin. Only a few penetration studies with nanoparticles on compromised skin have been conducted. Titanium-dioxide (TiO_2) and zinc-oxide (ZnO) nanoparticles in sunscreens were investigated to determine if nanoparticles in different formulations can penetrate through ultraviolet-B (UVB) damaged skin. Pigs, exposed to UVB that resulted in moderate sunburn, were used as a model for human skin. Both in vitro and in vivo studies were conducted with four sunscreen formulations: 10 %-coated-TiO_2-in-oil/water (o/w), 10 %-coated-TiO_2-in-water/oil (w/o), 5 %-coated-ZnO-in-o/w, and 5 %-uncoated-

ZnO-in-o/w. Penetration occurred more with the TiO_2 formulations than the ZnO formulations in both types of studies, but no absorption of TiO_2 or ZnO into the perfusate was noted by ICP-MS (Monteiro-Riviere et al. 2011b). Very little research has been conducted with diseased skin. Healthy and psoriatic skin depicted Ti deeply penetrated the stratum corneum of psoriatic skin relative to normal skin, but did not reach the viable epidermis (Pinheiro et al. 2007).

24.3.6 In Vivo Toxicity

The "gold standard" for assessing nanomaterial dermatototoxic potential is an in vivo study. The model systems employed to assess nanomaterial toxicity are identical to those used to assess chemical and drug skin irritation and toxicity, but nanomaterial studies have been limited. Regulatory agencies worldwide have specific prescriptive trial designs for conducting and interpreting dermal safety studies. Markers of in vivo skin toxicity include standard visual scoring indices such as the classic Draize test, evaluation of the barrier integrity using TEWL, or classic histopathology to assess microscopic lesions. The advantage of such systems is that identical exposure scenarios that would be encountered in humans can often be duplicated. The major limitation is that few chronic exposure studies have ever been conducted for any nanomaterial in any animal model, certainly not in humans. In vivo studies were conducted daily for 14 days using 25 nm and 35 nm carbon-coated silver nanoparticles (AgNP), as well as freshly synthesized or thoroughly washed 20 nm, 50 nm, and 80 nm AgNP topically applied to the backs of weanling pigs. The penetration of AgNP was only observed in the superficial layers of the stratum corneum after 14 days by TEM and confirmed by EDS which detected the localization of Ag (Samberg et al. 2010). Also, pristine hydrophobic fullerenes (C_{60}) were applied in a series of organic solvents or mineral oil to pigs for four days (see under solvents section) and showed that chloroform resulted in the deepest C_{60} stratum corneum penetration compared to the other solvents (Xia et al. 2010a).

24.4 What Characteristics of Nanomaterials Have Been Assessed Relative to Skin Penetration and Dermatototoxic Potential?

Table 24.1 lists the physicochemical properties most often used to characterize nanomaterials in the literature. Measuring these properties provides a benchmark for describing what nanomaterials were actually studied relative to individual particle size, shape, and surface chemistry including charge (Zeta potential). Of particular importance is the analysis for contaminants that may persist from the manufacturing process that provide a toxicological signature not the nanoparticle but of its method of synthesis since the adverse effects seen may be related to the contaminant introduced during synthesis rather than the nanoparticle.

However, the physical and chemical properties that are important in assessing biological interactions are those properties that reflect nanomaterial structure and function in the aqueous biological environment at physiologically compatible temperatures, ion concentrations, and pH. For the skin, one could argue those properties encountered on the skin surface or within the hydrophobic domains of the stratum corneum barrier. Ideally, nanoparticle properties should be measured in situ, but most often instead are measured under controlled laboratory conditions that may not reflect the biological milieu. The major exception is the use of scanning and primarily transmission electron microscopy to image

Table 24.1 The physicochemical properties of nanomaterials

Size distribution
Aggregation/agglomeration state
Shape
Crystal structure
Chemical composition
Surface area
Surface chemistry
Surface charge
Porosity

nanoparticles in cells and in tissues. The major limitation here is that only the electron dense components of nanoparticles can be imaged. The problem with non-biological nanomaterial characterization is that many of their properties (size, surface chemistry, shape, aggregation/agglomeration state) are sensitive to the environment they are in. Does aggregation or agglomeration dramatically alter particle size? When particle size is reported, is this the size of the individual non-aggregated particle or the size of the aggregated/agglomerated complex? These issues of characterization are now becoming more widely recognized and hopefully will result in the development of metrics and indices that reflect nanomaterial function in biologically relevant matrices.

Our laboratory has attempted to measure a potential molecular interaction fingerprint of the surface of a nanoparticle measured in an aqueous system (Xia et al. 2010b, 2011). We have defined the Biological Surface Adsorption Index (BSAI) as a five-component nanodescriptor fingerprint that quantitates the surface adsorption forces (hydrophobicity, hydrogen bonding, polarity/polarizability, and lone par electrons) that govern nanomaterial interaction with biological components that lead to aggregation, protein corona formation, and interactions of nanomaterials with biological systems such as cellular transport and trafficking domains. The properties assessed a broad classification of nanomaterials according to potential reactivity based upon descriptors that have been employed for generations to describe molecular interactions in the body (binding, partitioning, etc.). The advantage that BSAI type indices provide is that these properties can be simultaneously assessed for each nanoparticle type.

24.4.1 Quantum Dot (QD) Penetration and Toxicity Studies

We have studied nanoparticle penetration of QD and epidermal toxicity under controlled conditions in pigs using a series of QD of different sizes, shapes, and surface chemistries (Monteiro-Riviere and Baroli 2010; Ryman-Rasmussen et al. 2006; Zhang and Monteiro-Riviere 2008; Zhang et al. 2008). Penetration of QD565 (spherical) and 655 (elliptical) with a CdSe (cadmium selenide) core and a ZnS (zinc sulfide) shell dispersed in borate buffer with diverse physicochemical properties was studied in porcine skin flow-through diffusion cells. QD565 coated with polyethylene glycol (PEG) (35 nm; neutral), PEG-amine (NH_2) (15 nm; cationic), or carboxylic acid (COOH) (14 nm; anionic) showed penetration into the stratum corneum layers and localization within the epidermal and dermal layers by 8 h. PEG (45 nm) and PEG-NH_2 (20 nm)-coated QD655 were localized within the epidermal layers by 8 h. The penetration of QD655-COOH (18 nm) into epidermal layers was evident only after 24 h (Ryman-Rasmussen et al. 2006). In addition, QD621 (nail shaped) (39–40 nm) with a CdSe core and a CdS shell dispersed in water were topically applied to assess penetration in the same system. After 24 h, at the low concentration of 1 μM, QD621 was located primarily on the surface of the stratum corneum layers of the skin, and no QD621 fluorescence was detected in the stratum granulosum, stratum spinosum, or stratum basale layers of the epidermis. However, by increasing the applied concentration to 10 μM, QD621 was detected in the upper stratum corneum bilipid layers or in between the stratum granulosum-stratum corneum interface, with only a small amount of fluorescence in the upper viable epidermal layers (Zhang et al. 2008). Transdermal flux of any of these diverse QDs was not detected in perfusate.

The penetration seen with QD565, QD655, and QD621 in flow-through diffusion porcine skin cells showed how differences in composition, size, configuration, surface charge, dispersing medium, and other physicochemical parameters could influence penetration. PEG-QD621 with a hydrodynamic diameter of 39 ± 1 nm was capable of penetrating only the uppermost layers of the porcine stratum corneum after 24 h of exposure (Zhang et al. 2008), while confocal microscopy showed that all three surface coatings of the QD565 penetrated at 8 and 24 h, but only the QD655 COOH took 24 h to penetrate porcine skin (Ryman-Rasmussen et al.

2006). QD that are synthesized with the same core/shell and similar surface coatings and hydrodynamic diameters but different shapes and different penetration rates can penetrate intact skin. These studies showed that spherical QD penetrated better than the ellipsoid, cationic better than anionic across shapes, and all better than the nail-shaped QD 621 when compared to neutral QD with similar hydrodynamic dimensions. These results are not unexpected if one considers the skin architecture and dimensions of the penetration paths. Nevertheless, one could argue that the vehicle-mediated alterations in the skin cannot be completely ruled out; the penetration effects reported still remain valid for occupational considerations because the dosing solutions are representative of QD as commercially supplied, but at twofold to fourfold lower concentration. These QD studies clearly show independent effects of size, surface chemistry, and shape on stratum corneum penetration into a defined in vitro skin model. Similar studies with other nanomaterials using the same skin penetration model need to be conducted.

24.5 Summary

The study of the interaction of nanomaterials and their penetration through the skin has turned out to be a fruitful area of investigation: both as a unique interface for studying nanoparticle-tissue interactions and because of the real potential for human exposure from occupational, medicinal, consumer product, and cosmetic perspectives. From a pharmaceutical and pharmacological perspective, it appears unlikely that transdermal delivery of intact nanoparticles at pharmacologically significant fluxes into the systemic circulation will occur. Use of topical dermatological medicinal preparations for surface activities (e.g., sunscreens), hair follicle delivery, or potentially vaccinations seems feasible.

From a human safety perspective where large skin surfaces could be exposed to high exposure levels, for example, in cosmetics or sunscreens, the skin becomes an important organ for assessment. In most cases, classic irritation might be the only sequela observed, and this could be due to solvent or nanoparticle contaminant effects. Large transdermal fluxes are not required for toxicological effects to be initiated if the endpoint is sensitization or neoplastic transformation. This would be especially true if the skin barrier was disrupted by mechanical perturbation or disease.

What is lacking in this field is longer-term in vivo trials with fully characterized nanomaterials that have already been investigated using accepted and validated in vitro cell culture and skin penetration systems. As discussed above, many in vitro systems have inherent limitations due to factors such as nanomaterial interference with viability and cytokine assay biomarkers or nanomaterial interactions with biomolecules in vitro that are fundamentally different than what would be encountered in vivo. Presently, we do not have sufficient data available to reliably extrapolate in vivo behavior from in vitro data.

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