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Solid Lipid Nanoparticles: Technological Developments and in Vivo Techniques to Evaluate Their Interaction with the Skin

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1. Introduction

Nanotechnology is an emerging science involving manipulation of matter at the nanometer scale [Stern et al, 2008]. Nanoscience research has shown remarkable growth over the past 10 years, which is expected to continue for the foreseeable future. Discovery, development and implementation of nanotechnologies are driven by the social desire for smaller products with enhanced capabilities. As such, nanotechnology research and development has been increasing steadily. The discovery and development of nanotechnology is evident in food products, pesticides, consumer products and medicine. The application of nanotechnology to medicine is termed nanomedicine, which spans from nanodiagnostics to nanorobotic treatments and novel nanoparticle drug-delivery systems. The most prominent use of nanoparticles in medicine is the development of novel drug-delivery systems. The use of nanotechnology in drug delivery could revolutionize current therapies and is set for rapid advancements. This is due to the unique properties of nanomaterials, including large surface:mass ratio (i.e., large functional surface), ease in engineering tissue-targeted nanoparticles, and higher loading capacity due to reduced drug expulsion during storage compared with micro-sized systems, which increases their ability to carry natural and synthetic chemical compounds [Buse et al, 2010].

Although opportunities to develop nanotechnology based efficient drug delivery systems extend into all therapeutic classes of pharmaceuticals, the development of effective treatment modalities for the respiratory, central nervous system and cardiovascular disorders remains a financially and therapeutically significant need. Many therapeutic agents have not been successful because of their limited ability to reach to the target tissue. In addition, the faster growth opportunities are expected in developing delivery systems for anti-cancer agents, hormones and vaccines because of safety and efficacy shortcomings in their conventional administration modalities. For example, in cancer chemotherapy, cytostatic drugs damage both malignant and normal cells alike. Thus, a drug delivery strategy that selectively targets the malignant tumor is very much needed. Additional problems include drug instability in the biological milieu and premature drug loss through

rapid clearance and metabolism. Similarly, high protein binding of certain drugs such as protease inhibitors limits their diffusion to the brain and other organs. However, nanotechnology for drug delivery applications may not be suitable for all drugs, especially those drugs that are less potent because the higher dose of the drug would make the drug delivery system much larger, which would be difficult to administer [Sahoo et al, 2003].

The Royal Society and Royal Academy of Engineering define nanoparticles as particulate matter that has a size of 100 nm or less, while British Standards (British Department of Trade and Industry) define nanoparticles based upon the point at which the properties of nanoparticles differ from the bulk material – typically particulate with a scale less than 100 nm [[Buse et al, 2010]. However, not all particles used in drug delivery comply with this definition. Medical formulations of 100–500 nm are also considered nanoparticles since they share the same functionality in pharmaceutical applications and are governed by quantum effects instead of Newtonian physics [Bawa , 2005].

One nanoparticle class that has been widely used in drug delivery is lipid-based nanoparticles. Compared to liposomes and emulsions, solid particles possess some advantages, e.g. protection of non-incorporated active compounds against chemical degradation and more flexibility in modulating the release of the compound. Advantages of liposomes and emulsions are that they are composed of well tolerated excipients and they can easily be produced on a large scale, the pre-requisite for a carrier to be introduced to the market. At the beginning of the 1990s, the advantages of solid particles, emulsions and liposomes were combined by the development of the 'solid lipid nanoparticles' (SLN) [Muller et al,2002; Bunjes et al, 2006].

Solid lipid nanoparticles protect the drug from the environment and prevent its degradation whilst increasing its bioavailability. Therefore, two direct benefits can be achieved; first, due to the improved encapsulation efficiency, less of the active drug is required during the formulation process. Second, the desired effects of the drug are expedited due to an increase in the initial release, which is a result of the homogenization production process of SLNs. Both heating the lipid/water mixture and increasing the total surfactant concentration causes a partitioning of the active drug into the water phase due to an increase in its water solubility. During cooling, the solubility of the drug in water is decreased, allowing for the drug to partition back into the lipid phase [Üner, 2006]. The crystallization of the lipid core will occur while a high percentage of the drug is in the water phase. As a result, a considerable amount of the drug is accumulated on the surface of SLNs, hence the initial burst release of the drug after administration. The increase in the initial drug release can be offset by performing homogenization at lower temperatures (i.e., using cold homogenization) preventing drug expulsion and crystallization during cooling or using a greater amount of surfactant, which improves the lipid solubility of the drug entities [Buse, 2010]. This chapter describes the more innovative methods to produce and to characterize SLN and to assure their safety after cutaneous application.

In fact, after skin application of SLN, a lipidic film onto the skin is formed. Such a film can have an occlusive effect that increases drug penetration or increases water content in the upper epidermis layers.

Potential systemic effects after dermal application of SLN should be considered in order to obtain a safe topical product. Skin penetration and systemic absorption should be estimated with the intention to assess the risk of using nanoparticles in topical products. Visualization of colloidal systems after skin application is essential to evaluate their interaction with

cutaneous structures.   This chapter provides informations about the non-invasive bioengineering techniques to study the SLN distribution and interaction on the skin.

2. Solid lipid nanoparticles

2.1 Ingredients

General ingredients include solid lipid(s), emulsifier(s) and water. The term lipid is used here in a broader sense and includes triglycerides (e.g. tristearin, tricaprin, tripalmitin, partial glycerides (e.g. Imwitor, glycerol behenate), fatty acids (e.g. stearic acid), steroids (e.g. cholesterol) and waxes (e.g. cetyl palmitate) [Buse, 2010, Choi et al, 2008, Chen et al, 2006, Harivardhan et al, 2006].

A clear advantage of SLN with respect to polymeric nanoparticles is that the lipid matrix is made from physiological lipids which decrease the danger of acute and chronic toxicity. The vast majority of solid lipids are naturally occurring lipids (performing various physiological functions) and as a result they have lower cytotoxicity than synthetic polymers.

Critical parameters for nanoparticle formation will be different for different lipids. Examples include the velocity of lipid crystallization, the lipid hydrophilicity (influence on self-emulsifying properties) and the shape of the lipid crystals (and therefore the surface area). It is also noteworthy, that most of the lipids used represent a mixture of several chemical compounds. The composition might therefore vary from different suppliers and might even vary for different batches from the same supplier. However, small differences in the lipid composition (e.g. impurities) might have considerable impact on the quality of SLN dispersion (e.g. by changing the zeta potential, retarding crystallization processes etc.). For example, lipid nanodispersions made with cetyl palmitate from different suppliers had different particle sizes and storage stabilities.

All classes of emulsifiers (with respect to charge and molecular weight) have been used to stabilize the lipid dispersion (e.g., poloxamers, Tween 80, soya lecithin and sodium dodecyl sulphate). The choice of the emulsifiers and their concentration is of great impact on the quality of the SLN dispersion. It has been found that the combination of emulsifiers might prevent particle agglomeration more efficiently. The choice of the emulsifier depends on the administration route and is more limited for parenteral administrations. High concentrations of the emulsifier reduce the surface tension and facilitate the particle partition during homogenization. The decrease in particle size is connected with a tremendous increase in surface area. Therefore, kinetic aspects have to be considered [Uner, 2006].

Recently, the incorporation of a small amount of liquid lipids led to a new nanoparticle structures, the Nanostructured Lipid Carriers (NLC) in which the liquid lipid phase can be embedded into the solid matrix or to be localized at the surface of solid particles [Schafer-Korting et al, 2007].

The simplistic production methods and structural composition of SLNs has increased the appeal for their use in pharmaceutical formulations. For example, the inclusion of lipids that have 'generally recognized as safe' (GRAS) status minimizes cytotoxic effects whilst maintaining the overall stability of the SLN structure. However, structural stabilization of SLNs requires the incorporation of surfactants. While some surfactants may increase the cytotoxicity, nontoxic surfactants (e.g., lecithin) can be utilized to maintain the desired low cytotoxicity of SLNs [Cevc, 2004].

2.2 Preparation methods

Many different techniques for the production of lipid nanoparticles have been described in the literature. These methods are high pressure homogenization [Liedtke, 2000; Jahnke, 1998; Wissing et al, 2004], microemulsion technique [Gasco, 1993; Gasco, 1997; Priano et al, 2007], emulsification-solvent evaporation [Sjöström & Bergenstahl, 1992], emulsification-solvent diffusion method [Hu et al, 2002; Trotta et al, 2003], solvent injection (or solvent displacement) method [Schubert, & Müller-Goymann, 2003], phase inversion [Heurtault et al, 2002], multiple emulsion technique [Garcy´-Fuentes et al, 2002], ultrasonication [Pietkiewicz, & Sznitowska, 2004; Puglia et al, 2008] and membrane contractor technique [Charcosset et al, 2005].

However, high pressure homogenization technique has many advantages compared to the other methods, e.g. easy scale up, avoidance of organic solvents and short production time. High pressure homogenizers are widely used in many industries including the pharmaceutical industry, e.g. for the production of emulsions for parenteral nutrition. Therefore, no regulatory problems exist for the production of topical pharmaceutical and cosmetic preparations using this production technique. It can be considered as being industrially the most feasible one.

Lipid nanoparticles can be produced by either the hot or cold high pressure homogenization technique. In the hot homogenization method the lipid melt containing the active compound is dispersed in a hot surfactant solution of the same temperature by high speed stirring. The obtained emulsion (generally called pre-emulsion) is then passed through a high pressure homogenizer adjusted to the same temperature. In the cold homogenization method, the active containing lipid melt is cooled down. After solidification the mass is crushed and ground to obtain lipid microparticles. The lipid microparticles are then dispersed in a cold surfactant solution yielding a cold pre-suspension of micronized lipid particles. For hot and cold homogenization is not required the use of organic solvents, in this way the cytoxicity is further reduced.

2.3 SLN characterization

After preparation, it has to be ensured that the particles obtained have the desired properties and are thus suitable for the intended type of administration. The most obvious parameters to be investigated are the (colloidal) particle size and the (solid) state of the particle matrix. Other important features include the surface characteristics, the particle shape, and in particular, the interaction with incorporated drugs. Due to the complexity of the systems, a combination of different characterization techniques is the most promising approach to obtain a realistic image of the sample properties [Pardeike J. et al, 2009].

Amongst the methods used to measure particle size, Photon Correlation Spettroscopy (PCS) is the most widely employed. Particle size of the SLNs is influenced by different factors such as lipid matrix, drug to lipid ratio, surfactant blend, viscosity of lipid and aqueous phase and production parameters.

The zeta potential is used as a measure of surface charge. This is valuable in preventing aggregation and imparts the physical stability to formulation. At higher zeta potential, particle aggregation is less likely to occur, due to electrical repulsion.

Shape and Morfology of lipid nanoparticles were usually investigated using Scanning Electron Microscopy (SEM), Transmission Electron Microscopy (TEM) and Atomic Force Microscopy (AFM).

The cristallinity and the polymorphic behavior of lipids strongly influence drug incorporation and release rate. Crystalline solids lipids have an olderly arrangement of units. The presence of emulsifiers, the preparation method, and the high dispersity as well as the small particle size of the colloidal system influences crystallinity of lipids in the SLNs. Basic techniques used to investigate of the lipid are Differential Scanning Calorimetry (DSC) and X-Ray Diffractometry (XRD) [Rizwan et al, 2009].

About the encapulation capability there are basically three different models for the incorporation of active ingredients into SLN: homogeneous matrix model, drug-enriched shell model and drug-enriched core model. The structure obtained is a function of the formulation composition (lipid, active compound, surfactant) and of the production conditions (hot vs. cold homogenisation)[Mehnert&Mader, 2001, Haskell, 2006].

Solid lipid nanoparticles (SLN) are able to incapsulate a great amount of lipophilic compounds such as steroids, retinol, sunscreens but for most drugs, especially hydrophilic ones, the payload is very low. This effect can be due either to the crystalline structure of the lipid matrix, or to the low solubility of hydrophilic substances into lipidic phase.

Two approaches were developed to improve the payload of hydrophilic compounds. The first approach was the development of oil loaded SLN, also described as nanostructured lipid carriers (NLC). The alternative strategy, applied recently from Perugini et al., is to modify the lipid matrix by incorporation of amphiphilic substances such as phosphatydilcholine, polyglyceryl-3-diisostearate and sorbitan [Perugini et al, 2010b].

In addition to usual particle characterization, to ensure the safety of topical preparations *in vitro* cytotoxicity of SLN should be evaluated. Cellular damage results in loss of the metabolic cell function. The tetrazolium salt MTT, using keratinocytes line, is widely used to quantitate by colorimetric assay the cytotoxicity of preparations. The tetrazolium salts are metabolically reduced to highly colored end products called formazans. The colorless MTT is cleaved to formazan by the succinate-tetrazolium reductase system which belongs to the mitochondrial respiratory chain and is active only in viable cells. [Weyenberg et al, 2007]. Perugini et al. calculated, by linear regression analysis of data, the EC50 values of SLN aqueous suspension in order to define non toxic concentration of nanoparticles suspension that can to be use in topical formulation [Perugini et al, 2010a].

Furthermore, the two most frequent manifestation of skin toxicity are irritant contact dermatitis and allergic contact dermatitis. Keratinocytes, which represent the 95% of epidermal cells in both human and mouse skin, are a rich source of cytokines and they actively participate to skin inflammatory and immunological reactions. Among the cytokines produced by keratinocytes, IL-1 is one of the most interesting, since it is produced constitutively by keratinocytes and retained in normal conditions into the cell. In general, cytokines have been identified as useful tools to discriminate between irritant and allergic contact dermatitis [Corsini et al, 1998]. Perugini et al. using a specific sandwich ELISA to evaluate IL-1 release from keratinocytes after SLN application [Perugini et al, 2010].

3. Skin

The skin (Latin: cutis) consists of 250 Am (<4000 Am) thick inner skin region (=dermis), and of 50 Am (<100 Am) thick outer skin region (=epidermis). The fact that the skin is one of best biological barriers is mainly due to the outermost part of the epidermis, the skin horny layer (Latin: the stratum corneum, SC) [Walters, 2002].

The SC serves as the principal barrier against the percutaneous penetration of chemicals and microbes and is capable of withstanding mechanical forces [Madison, 2003]. It is further involved in the regulation of water release from the organism and into the atmosphere, known as transepidermal water loss (TEWL).

The SC forms a continuous sheet of protein-enriched cells (corneocytes) connected by corneodesmosomes and embedded in an intercellular matrix enriched in non-polar lipids and organized as lamellar lipid layers. The final steps in keratinocyte differentiation are associated with profound changes in their structure, resulting in their transformation into the flat and anucleated corneocytes of the SC, which are loaded with keratin filaments and surrounded by a cell envelope composed of cross-linked proteins (cornified envelope proteins) as well as a covalently bound lipid envelope. Extracellular non-polar lipids surround the corneocytes to form a hydrophobic matrix.

During the final stages of normal differentiation, keratins are aligned into highly ordered and condensed arrays through interactions with filaggrin, a matrix protein. The role of filaggrin in skin barrier homeostasis is only partially known. Filaggrin aggregates the keratin filaments into tight bundles. This promotes the collapse of the cell into a flattened shape, which is characteristic of corneocytes in the cornified layer [Palmer et al, 2006, Proksch et al, 2008]. Together, keratins and filaggrin constitute 80–90% of the protein mass of mammalian epidermis [Roop, 1995, Nemes &Steinert, 1999].

The cornified cell envelope (CE) is a tough protein / lipid polymer structure formed just below the cytoplasmic membrane and residing on the exterior of the corneocytes. It consists of two parts: a protein envelope and a lipid envelope. The protein envelope contributes to the biomechanical properties of the CE as a result of cross-linking of specialized cornified envelope structural proteins, including involucrin, loricrin, trichohyalin and to the class of small proline-rich proteins by both disulphide bonds and N-epsilon-(gamma-glutamyl)lysine isopeptide bonds formed by transglutaminases [Candi et al, 2005, Roop, 1995].

Desmosomes on corneocytes are called (corneo)desmosomes. Desmosomes, which connect the keratinocytes of the granular layer with the corneocytes of the SC, have been called transition desmosomes and are exclusively found at that location [Koch &Franke, 1994, Al-Amoudi et al, 2005]. Adjacently interconnected corneocytes are important for SC cohesion and are shed during the desquamation process in the SC. The corneocytes provide mechanical and chemical protection and, together with their intercellular lipid surroundings, confer water impermeability [Madison 2003]. The cohesiveness of, and the communication between, the viable epidermal cells, the cell-cell interaction, is maintained in a fashion similar to the cell-matrix connection, except that desmosomes replace hemidesmosomes. Adherens junctions are also located at keratinocyte-keratinocyte borders. At the desmosomal junction there are two transmembrane glycoprotein types: desmogleins and desmocollins wich are associated with the cytoplasmatic plaque proteins and provide a linkage to keratin intermediate filaments. On the other hand, in the adherens junction, classic cadherins act as transmembrane glycoproteines and these are linked to the actin filament α-, β- and χ-catenins. Thus, in the epidermis, the desmosomes are responsible for interconnecting individual cell keratin cytoskeletal structure, thereby creating a tissue very resistant to shearing forces [Prow et al, 2011].

The major lipid classes in the SC are ceramides, free fatty acids and cholesterol [Downing et al, 1987]. Ceramides are amide-linked fatty acids containing a long-chain amino alcohol called sphingoid base and account for 30 to 40% of SC lipids. The SC contains at least nine

different free ceramides [Huchida &Hamanaka, 2006], two of which are ceramide A and ceramide B, covalently bound to cornified envelope proteins, most importantly to involucrin [Bouwstra et al, 2006]. The epidermis contains free fatty acids as well as fatty acids bound in triglycerides, phospholipids, glycosylceramides and ceramides. The chain length of free fatty acids ranges from C12 to C24. Saturated and monounsaturated fatty acids are synthesized in the epidermis, while others must be obtained from food and blood flow. Cholesterol is the third major lipid class in the SC. Although basal cells are capable of resorbing cholesterol from circulation, most cholesterol in the epidermis is synthesized in situ from acetate.
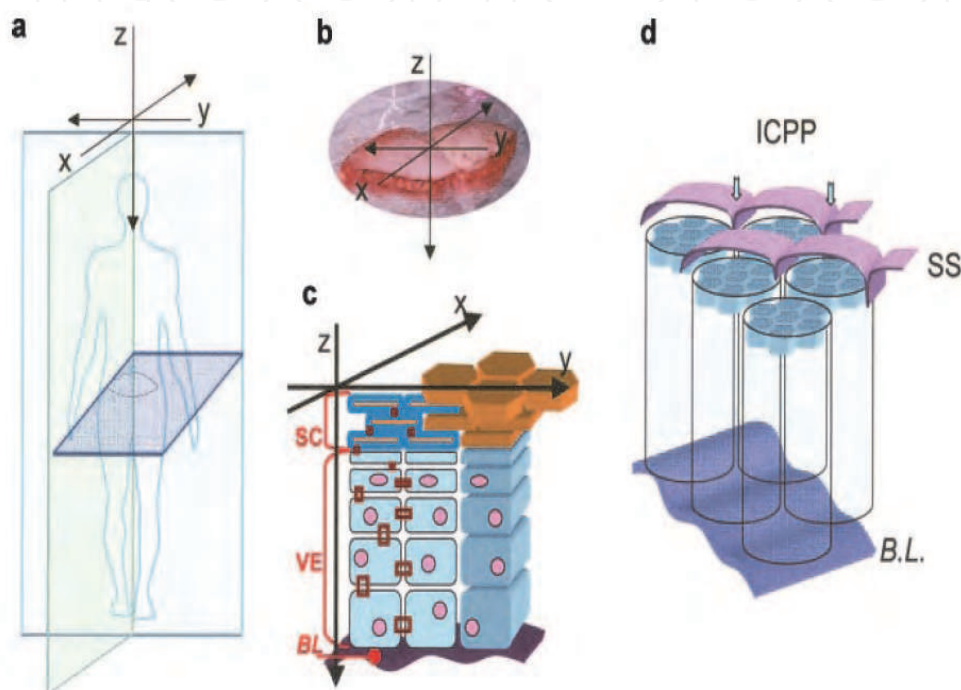
Fig. 1. Structure of epidermis. This figure shows (a) a human body representation in relation with the X–Y–Z coordinate system. The area of abdominal specimen origin is also shown (below the belly); (b) an abdominal skin specimen deposited flat on a table, where stratum corneum is upward and in the X–Y plane; (c) a schematic representation of epidermis in the Y–Z plane, where stratum corneum is SC, viable epidermis VE, and basal lamina B.L. Squares between keratinocytes and corneocytes respectively represent desmosomes and corneodesmosomes. Hexagonal shape of corneocytes is shown in the X–Y plane, where an attempt of a 3D perspective is provided. (d) Representation of corneocyte clusters and their relationships with basal lamina (B.L.) and skin surface (SS). Skin produces furrows along cluster perimeters, where the intercluster penetration pathway (ICPP) is supposed to exist[Baroli 2010]

There are still many unanswered questions about the exact way in which the SC lipids are organized at the molecular level and this is an active area of research [Bouwstra et al, 2003]. Understanding the physical structure of the membranes is critical to understanding their function as a barrier, both to water and to other substances, and ultimately to understanding the mechanisms of barrier disruption in a variety of skin diseases Numerous biophysical studies of SC structure suggest the presence of coexisting liquid crystalline and gel phase

domains in the membranes of the SC. This concept was suggested by Forslind (1994) [Forslind, 1994] and presented as the ''domain mosaic'' model; a new model for the existence of fluid phases within the lamellae, the ''sandwich model'', was presented by Bouwstra et al (2000) [Bouwstra et al, 2000]. Norlen, however, has proposed a different ''single gel phase'' model feeling more consistent with the documented barrier properties of the SC [Norlen 2001, Baroli 2010].

Although the SC is recognized as the most important physical barrier, the nucleated epidermal layers are also significant in barrier function. Clinical observations confirm the importance of the nucleated epidermal layers in skin barrier function by preventing both excessive water loss and the entry of harmful substances into the skin [Honari 2004]. Tight junctions (TJ) are cell–cell junctions which connect neighbouring cells, control the paracellular pathway of molecules (barrier function) and separate the apical from the basolateral part of a cell membrane (fence function). In human epidermis, various TJ proteins have been identified, including occludin, claudins 1, 4, and 7, JAM-1 (junctional adhesion molecule-1), zonula occludens protein 1 and MUPP-1 (multi-PDZ protein-1) [Brandner et al 2006a, Brandner et al 2006b]. In human skin, TJ proteins and/or discrete TJ-like structures are found in the interfollicular epidermis as well as in the skin appendages [Pummi 2001, Wilke et al 2006, Madison 2003].

3.1 Skin as a site for particle delivery

Skin is a widely used route of delivery for local and systemic drugs and is potentially a route for their delivery as nanoparticles. Whilst nanoparticle drug delivery has been touted as an enabling technology, its potential in treating local skin and systemic diseases has yet to be realised. Most drug delivery particle technologies are based on lipid carriers, i.e. solid lipid nanoparticles and nanoemulsions of around 300 nm in diameter, which are now considered microparticles.

The skin has historically been used for the topical delivery of compounds but it is only since the 1970s with the advent of transdermal patches that it has widely been used as a route for systemic delivery. Nanoparticle delivery to the skin is being increasingly used to facilitate local therapies. The nanoparticle definition designated by the National Nanotechnology Initiative has been adopted by the American National Standards Institute as particles with all dimensions between 1 nm and 100 nm. Fig. 2 shows that the potential sites for targeting nanoparticles include the surface of the skin, furrows, and hair follicles [Souto & Muller 2008]. A recent review by Baroli discusses nanoparticle penetration largely from the skin structure perspective debate of nanoparticle penetration [Baroli 2010, Sawant & Dodiya 2008, Medina et al 2007].

The theory and practical aspects of percutaneous penetration of drugs, particulate material and contaminants have been covered in a number of excellent reference texts. Souto and Muller maintained that submicron-sized particles show adhesiveness when in contact with surfaces. This property has been demonstrated for polymeric nanoparticles and for liposomes.

Regarding lipid nanoparticles, it has been published that approximately 4% of lipid nanoparticles with a diameter of approximately 200 nm should form theoretically a monolayer film when 4 mg of formulation is applied per cm². Being hydrophobic in character, this mono-layered film has an occlusive action on the skin retarding the loss of

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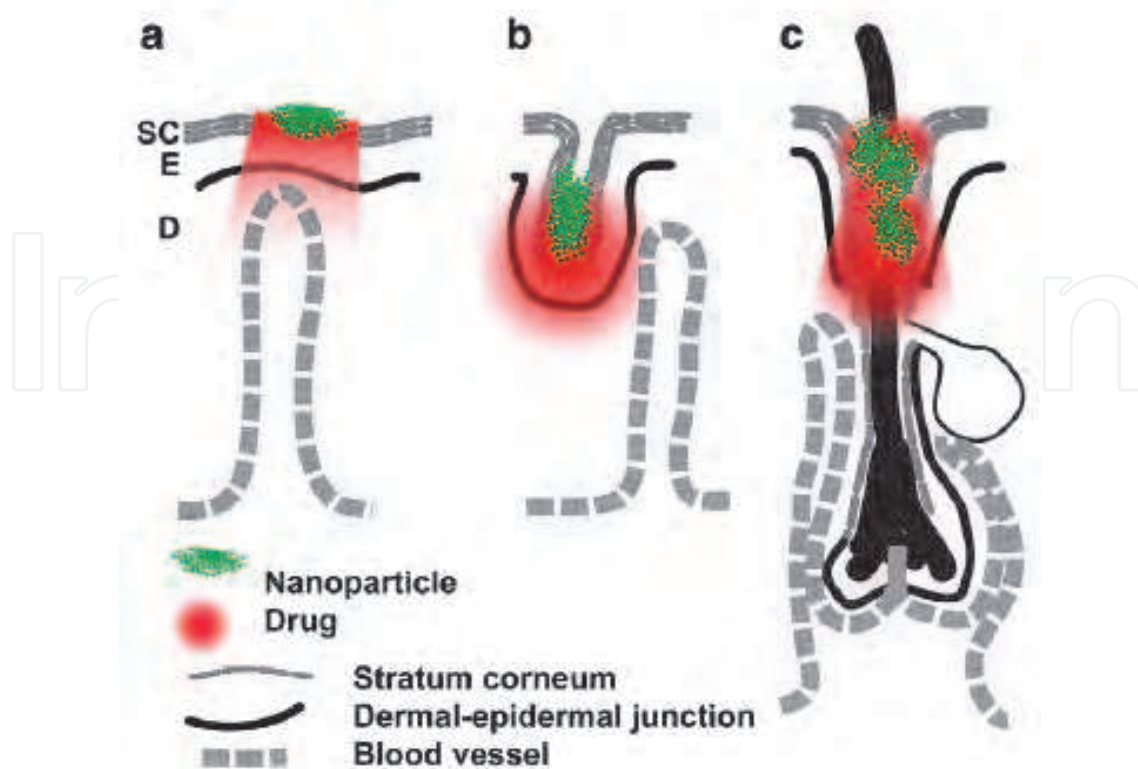and in Vivo Techniques to Evaluate Their Interaction with the Skin

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Fig. 2. Sites in skin for nanoparticle delivery. Topical nanoparticle drug delivery takes place in three major sites: stratum corneum (SC) surface (panel a), furrows (dermatoglyphs) (panel b), and openings of hair follicles (infundibulum) (c). The nanoparticles are shown in green and the drug in red. Other sites for delivery are the viable epidermis (E) and dermis (D)[Prow et al, 2011].

moisture caused by evaporation which can result in reduction of corneocyte packing and opening of inter-corneocyte gaps and thus facilitates the drug penetration into deeper layers of skin [Desai et al 2010, Wissing & Muller 2003]. The loss of water content from the SLN induces crystal modification of SLN matrix and this can induce drug expulsion and penetration [Lombardi Borgia et al 2005]. The occlusion effect of these nanoparticles is dependent on the applied sample volume, particle size, crystallinity and lipid concentration [Teeranachaideekul et al 2008]. The space filled with air in a layer of optimal packing density is independent on the particle size, which is considered to be 24% if assuming a three-dimensional hexagonal packing of ideal spherical-like particles [Desai et al 2010].

At the other hand the promise of nanoparticle-mediated drug delivery into the epidermis and dermis without barrier modification has met with little success. Where the barrier is compromised, however, such as in aged or diseased skin, there may be potential for enhanced particle penetration. Transport of substances across the SC occurs mainly by passive diffusion and based on the dual-compartment bricks and mortar structure of the SC, interrupted by appendages, is considered to occur via three possible routes. These are the transcellular, the intercellular and the appendageal routes. For most penetrants, the intercellular route is favoured. Small molecules are able to move freely within the inter-cellular spaces and diffusion rates are governed largely by their lipophilicity, but also physicochemical properties such as molecular weight or volume, solubility and hydrogen bonding ability [Potts & Guy 1995]. However, the free movement of macromolecules or

particles may be physically restricted within the lipid channels, which have been estimated by van de Merwe et al. to be 19 nm [Van der Merwe et al 2006] and by Baroli et al. to be 75 nm [Baroli et al 2007]. This suggests that for such materials, the SC could present an additional barrier that is not present for small molecules.

Polar and non-polar solutes were originally thought to permeate through the SC via separate routes [Scheuplein 1965], with polar solutes taking a transcellular route and more lipophilic solutes going via the intercellular lipids. However a perception of the difficulty of repeated partitioning between lipophilic and hydrophilic compartments in the SC led to this pathway being regarded as unlikely in most cases. There is considerable interest in targeted follicular delivery with tailored drug formulations [Grice et al 2010] or nanoparticle-bound drugs [Lademann et al 2007,Souto &Muller 2008].

Hair follicles were regarded as insignificant as potential routes for drug delivery, covering only 0.1% of the human skin surface area, their complex vascularisation and deep invagination with a thinning SC has led to a reappraisal of this view. Work has been done on assessing the contribution of the follicular route to drug penetration, as well as targeted delivery [Essa et al 2002, Teichmann et al 2006]. The deposition of these systems in hair follicles was observed by some authors and follicular penetration of solid particles has been demonstrated [Rolland et al 1993, Lademann et al 2009a]. Lademann discussed the finding that 300–600 nm particles penetrated follicles best on massage as a consequence of the distance between the scales on the hairs, and suggested that the movement of the hair acted as a geared pump to push the particles into the follicle [Lademann et al 2009b]. They viewed follicles as an efficient reservoir for nanoparticle-based drug delivery [Lademann et al 2007]. It is possible also that some follicles may be blocked by a "plug" of sebum or closed leading to particle penetration being impeded. Significant penetration of 40 nm nanoparticles beyond the follicles into epidermal cells can occur when the hair sheath has been pulled out [Vogt et al 2006, Ryman-Rasmussen et al 2007, Schroeter 2010, Muller et al 2002].

3.2 Toxicity of nanocarrier systems

Humans have been exposed to nanoparticles throughout their evolutionary phases; however, this exposure has been increased to a great extent in the past century because of the industrial revolution. The growing use of nanotechnology in high-tech industries is likely to become another way for humans to be exposed to intentionally generate engineered nanoparticles. However, the same properties (small size, chemical composition, structure, large surface area and shape), which make nanoparticles so attractive in medicine, may contribute to the toxicological profile of nanoparticles in biological systems [Muller 2000, Koo et al 2005, Vega-Villa et al 2008].

Because of increased use of nanotechnology, the risk associated with exposure to nanoparticles, the routes of entry and the molecular mechanisms of any cytotoxicity need to be well understood.

In fact, these tiny particles (<1000 nm in size) are able to enter the body through the skin, lungs or intestinal tract, depositing in several organs and may cause adverse biological reactions by modifying the physiochemical properties of living matter at the nanolevel [Muller 2000, Monteiro-Riviere & Baroli 2010].

The likelihood of nanoparticle penetration across the skin has recently been reviewed by the Scientific Committee on Consumer Products (SCCP) who conclude that in relation to dermal exposure:

- There is evidence of some skin penetration into viable tissues (mainly into the stratum spinosum in the epidermal layer, but eventually also into the dermis) for very small particles (less than 10 nm), such as functionalised fullerenes and quantum dots.
- When using accepted skin penetration protocols (intact skin), there is no conclusive evidence for skin penetration into viable tissue for particles of about 20 nm and larger primary particle size as used in sunscreens with physical UV-filters.
- The above statements on skin penetration apply to healthy skin (human and porcine). There is an absence of appropriate information for skin with impaired barrier function, e.g. atopic skin or sunburned skin. A few data are available on psoriatic skin.
- There is evidence that some mechanical effects (e.g. flexing) on skin may have an effect on nanoparticle penetration.
- There is no information on the transadnexal structures penetration for particles under 20 nm. Nanoparticles of 20 nm and above penetrate deeply into hair follicles, but no penetration into viable tissue has been observed.

The statement that nanoparticles of 20 nm in diameter do not penetrate into viable tissue is controversial. There have been reports showing nanoparticles 20 nm penetrating through the stratum corneum(SC), to the viable epidermis. The reasons for this important disparity may be due to differences in nanoparticle constituents, models, and methodologies [SCCP opinion 2007].

4. Evaluation of nanoparticles interaction with skin

Among the different methods that exist for the evaluation of the efficacy of topical products, the non-invasive biophysical measurements have the advantages of being non-invasive, non traumatizing, causing minimal discomfort and not altering the skin function. In addition, they permit to detect defined parameters, which in most cases cannot be discriminated by visual scoring [Berardesca et al 1995].

In order to evaluate nanoparticles interaction with the skin and their efficacy after cutaneous application, a number of techniques can be involved, namely:

- Assessment of the cutaneous electrical properties and of the water evaporation through the epidermis (TEWL) in relation to the water content of the outer skin layers;
- Evaluation of the mechanical/visco-elastic properties of the skin (measurement of the skin reaction to friction, torsion and suction)
- Instrumental evaluation of skin topography: surface/texture and desquamation (digital image analysis, silicone replicas and profilometry)
- Analyzing some spectroscopic and optical properties of the skin (ultrasound, near infrared and Raman spectroscopy, confocal microscopy).

4.1 Electrical and mechanical properties of the outer skin layers
4.1.1 Stratum corneum hydration and transepidermal water loss

Skin capacitance represents one of the most popular method to assess the water content in the stratum corneum by an indirect way, rising with the increase of skin hydration/moisturization. However, the relationship between this parameter and the water content of the skin is not linear but rather more complex, as other factors such as ions, the dipolar structure of protein as well as the different strength of water binding to keratin or the presence of hair on the skin surface influence the measurement.

Until now, only a few works are performed on the "in vivo" evaluation of solid lipid nanoparticles efficacy after topical administration and all of them demonstrated that an increase of stratum corneum (SC) hydration was obtained after SLN application [Muller et al 2002, Muller et al 2007].

The mechanism with which nanoparticles can produced this effect could be by the formation of an occlusive film on the skin, by the restoration of the skin lipid barrier or by a humectant effect retaining the water of the formulation avoiding the water evaporation from the deeper layers of the skin. The simultaneous measurement of the transepidermal water loss (TEWL) is very important in order to understand the real mechanism of nanoparticles.

In general, in the healthy skin, there is proportionality in the relation between TEWL and SC hydration. A decrease in TEWL, parallel with an increase in the stratum corneum hydration, is observed after the application of occlusive substances (petrolatum) on the skin. Elevated TEWL is detected immediately after application of moisturizing agents as a consequence of the evaporation of the water incorporated in the cosmetic product and it is not due to the increase in the SC hydration [Darlenski & Fluhr 2011]. For these reasons, a combination of more non-invasive technique is advised as more reliable and accurate approach.

4.1.2 Assessment of the skin mechanical properties

Skin mechanical properties are changing with aging in relation to the alterations of the dermal tissue composition [Smalls et al 2006]. The in vivo mechanical properties of the skin were studied by different methods based mainly on torsion stress and suction.

One of the most common instrument used in dermatological and cosmetic fields is the Cutometer (Courage&Khazaka electronic, Cologne , Germany) equipped with a 2 or 6 mm measuring probe. The time/strain mode used consists with a 2-s application of a constant negative pressure of 500 mbar, followed by a 2-s relaxation period. Each measurement consisted of three consecutive cycles. A typical skin deformation curve is illustrated in Figure 3.
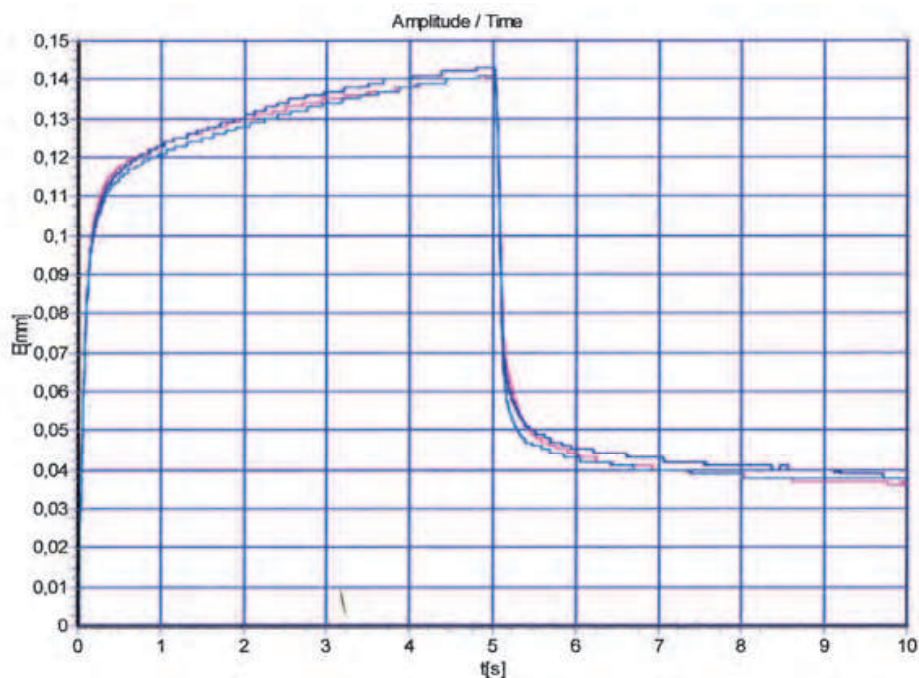

Fig. 3. Typical skin deformation curve

From these curves the following parameters were analyzed: R0 final distension (skin distensibility), R2 skin elasticity including viscous deformation and R5 skin elasticity without viscous deformation.

Until now, only a few work studied the influence of SLN application in changes of skin mechanical properties [Muller et al 2002, Muller et al 2007].

4.2 Instrumental evaluation of the skin topography

The examination of the skin surface topography with its scaling and roughness can be performed by several non-invasive techniques.

4.2.1 Replica

Many testing procedures used to examine the skin require biopsies or some other surgical manipulation. Skin replica is a technique used for the indirect topographic measurement of the skin and the procedure consists in placing a thin layer of silicone based resin on test skin surface in order to obtain an imprint [Ryan et al 1983, Hatzis 2004].

Replica technique is a non-invasive procedure developed for replicating human skin. Skin replica is simple, accurate and allows for records of skin condition to be kept [Hatzis 2004]. Nevertheless the use of this technique requires systematic methodology development and rigorous adherence to experimental protocol, as artefacts can be readily introduced [Ryan et al 1983]. Therefore, the quality of replicas is a limiting factor for the accuracy of the visualization. If the silicone is spilled over the borders of the ring or if the ring's thickness varies or if air bubbles are present in the gel the measurement results are heavily influenced. For these reasons is fundamental to chose a fluid material enough to penetrate the furrows, but not excessively fluid as to avoid overflowing outside the interested area. Furthermore a constant pressure of about the gel applied on the skin must be applied in order to obtain replicas with reproducible thickness [Bogner et al 2007].

Perugini et al demonstrated how the association of skin replica and Scanning Electron Microscopy (SEM) can be used for in vivo visualization of solid lipid nanoparticles on the skin surface [Perugini et al, 2011]. The results highlighted that particle with different matrix composition had a different behaviour. They studied two SLN suspension: the first contained phosphatidylcholine in the lipophilic phase of nanoparticles (BR1) while the second formulation contained cetearyl glucoside, a nonionic surfactant (TE1). The last formulation is, therefore, more apolar and could be more similar to the silicone material of the replica (Figure 4), for this reason nanoparticles seem to be on surface or incorporated into the replica material.

In the same study the behaviour of lipid systems applied on the skin of volunteers with different age was investigated. In the case of the child skin (8 years old) the particles seem to be distributed mainly on the microrelieves of the silicone resina: primary and secondary lines of the skin [Hashimoto 1974]. The behaviour changes when we observe with the same magnification the replica of the woman (38 years old). In this replica the particles distribution seems more homogeneous and particles are present both in relieves and in furrows (Figure 5).

To explain these results it's useful to remember that skin relieves reflect the organization of the deeper layers of the skin and changes at the level of the dermis will affect skin relieves. Skin of a child and skin of a woman are very different. The number of primary and secondary lines decreases progressively with age increasing and the polygonal surface

delimited by these lines becomes less homogeneous and defined [De Paepe et al 2000]. These changes could be the reason of the different system distribution on the skin surface.
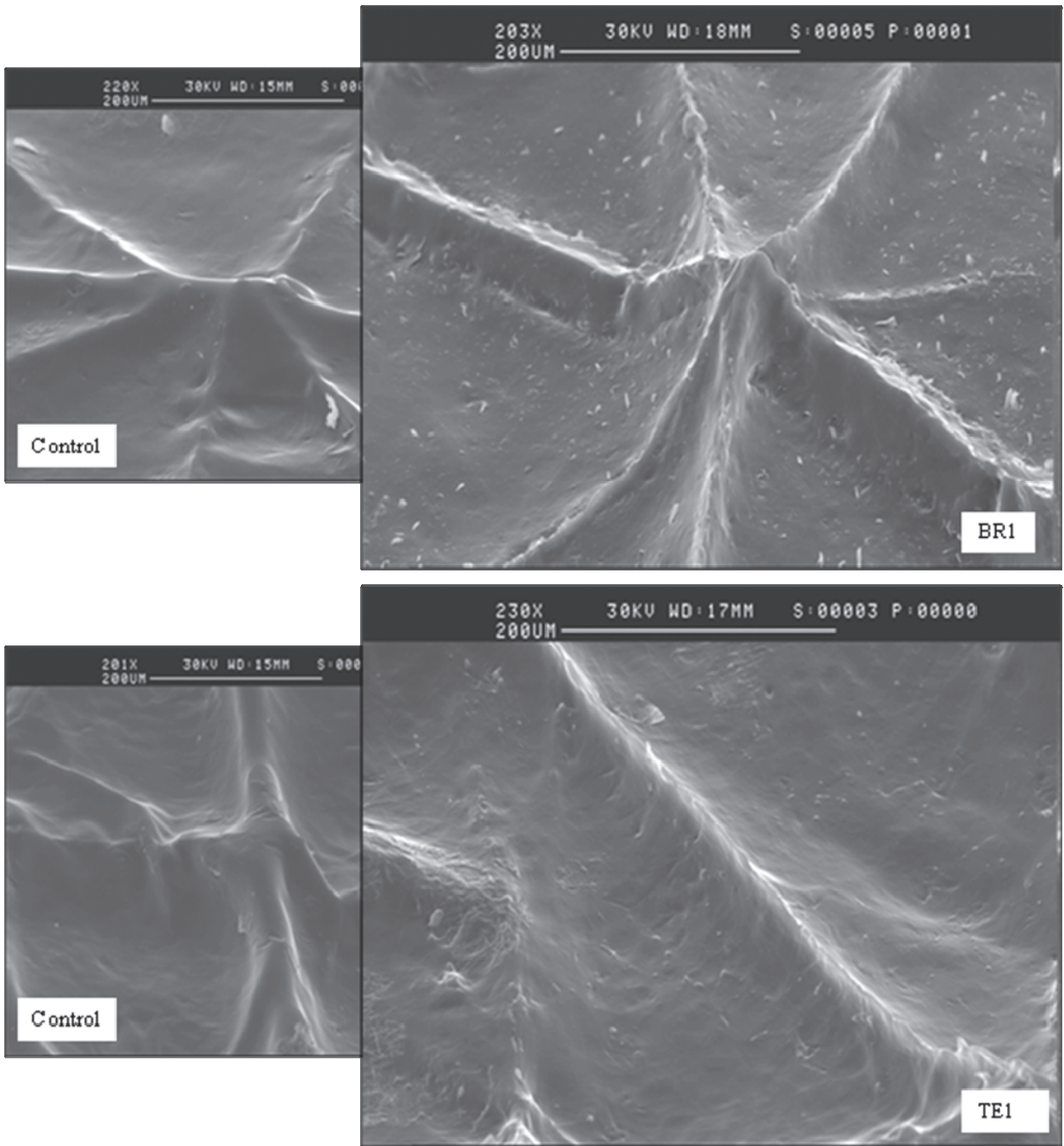

Fig. 4. Scanning electron micrographs of replicas obtained 1 h after solid lipid nanoparticle (batches BR1 and TE1) application (on the right) and the corresponding control replicas (on the left).
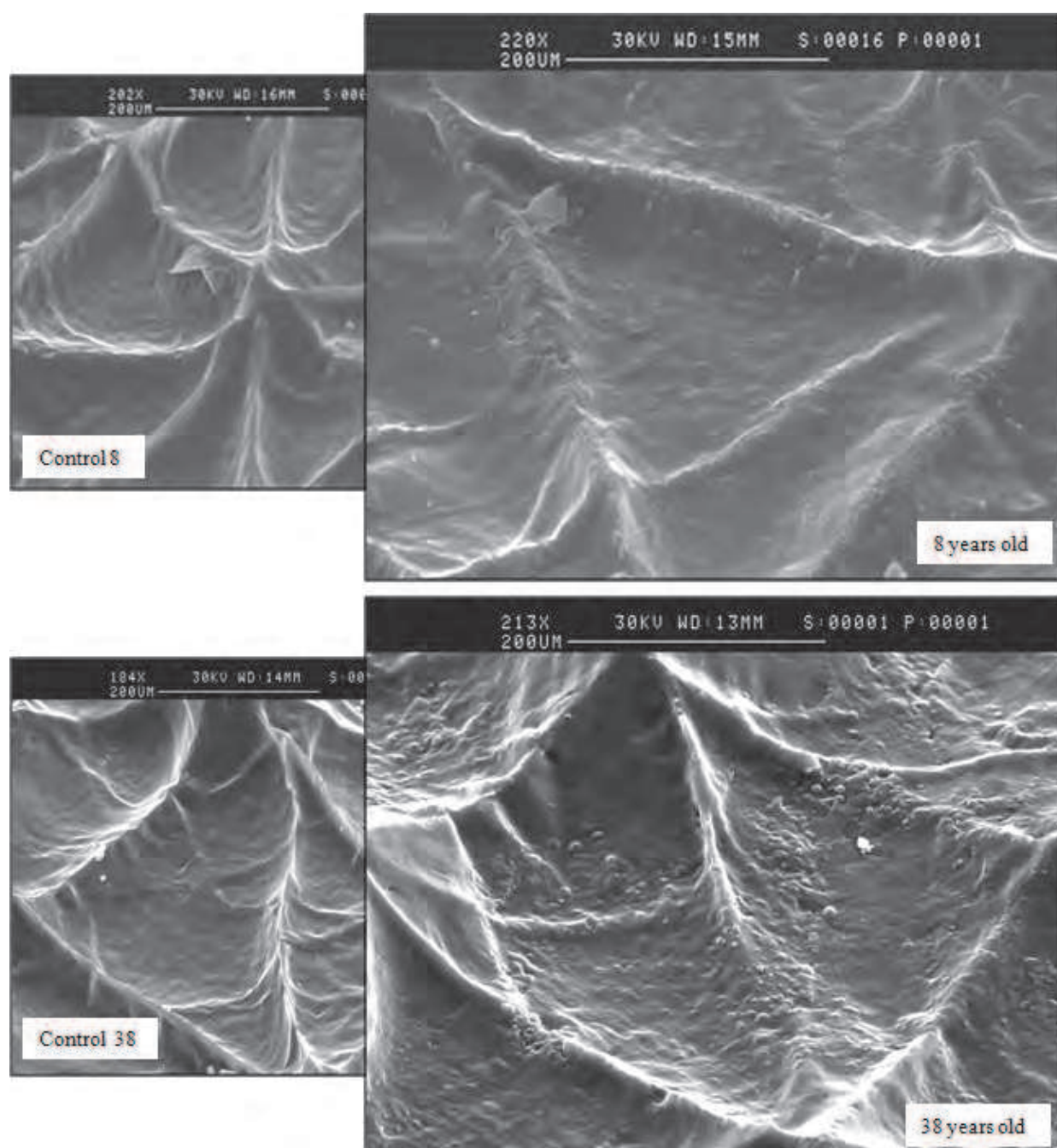
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Fig. 5. Scanning electron micrographs of replicas obtained after the application of TE
systems on the skin of an 8-year-old child and a 38-year-old woman.

4.2.2 Three-dimensional (3D) imaging of the skin

Three-dimensional (3D) imaging is a new measuring method in medicine. It uses optical
projections, a high resolution video camera, and computer software to rapidly generate
images and measurements of skin topography. The method has been used successfully in
wound care, dermatological laser treatments and its fundamental physics have been
validated.

The skin surface structure can to be analyze in the non-contact mode using the 3D optical
system Primos (GFMesstechnik GmbH, Teltow, Germany) as described in detail by Jacobi et

al. [Bloemen et al 2011]. This system is based on the digital stripe projection technique, which is used as an optical measurement process. A parallel stripe pattern is projected onto the skin surface and depicted on the CCD chip of a camera through an optical system. The measurement system consists of a freely movable optical measurement head (with an integrated micro-mirror projector, a projection lens system, and a CCD recording camera), together with an evaluation computer. The 3D effect is achieved by the minute elevation differences on the skin surface, which deflect the parallel projection stripes. The measurements of these deflections represent qualitative and quantitative measurements of the skin profile. The instrument determine the roughness, which is based on the depth and the density of the furrows and wrinkles of the skin.

The average roughness (Ra) is the mathematical average value of profile amounts within the total measuring length, and represents the roughness of the skin surface structure (Figure 6). High roughness values corresponded to deep furrows and wrinkles with a high density. This parameter is based on the sampling of all the points characterizing the profile, so it represents a true average because it is very significant in regard to the roughness of the skin.
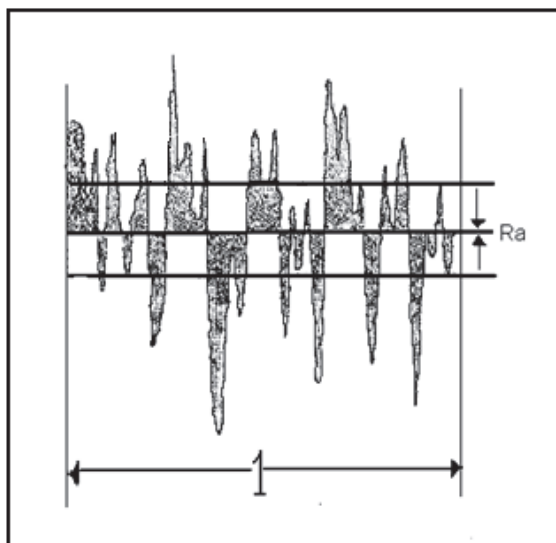

Fig. 6. Roughness mean value (Ra)

The Ra is two-dimensional parameter. For skin with very anisotropic characteristics, the value of this parameter can be very different depending on the direction along which the profile being analyzed (Figure 7). Ra has a higher value in perpendicular orientation to respect to the grooves (X direction in the figure 2); in fact, Rax> Ray. It must, therefore, to process simultaneously a large number of profiles that follow all possible directions and calculate the average of several measurements obtained.

The number of lines to be measured is an important factor; it is therefore very important to try as many lines as possible, to encompass the entire area of the captured image in the analysis; the higher the number of available profiles, the greater will be the precision of measurement.

Furthermore the Primos system evaluate, by matching, the same skin area in the different subsequent analysis times. 3D image of the skin, before and after SLN application, could be used to study SLN behavior. In order to evaluate the distribution of particle systems with different size on human skin, a 3D skin images before and after application of microparticles and nanoparticles formulations, were acquired.

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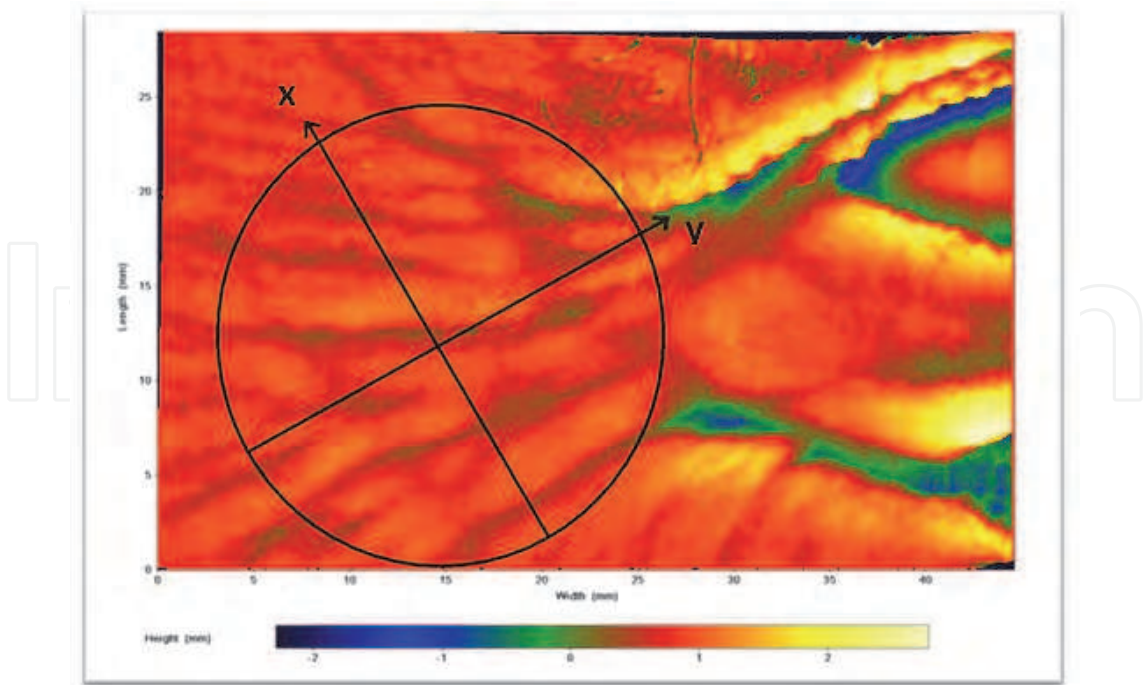and in Vivo Techniques to Evaluate Their Interaction with the Skin

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Fig. 7. Microrelief image. In particular directions along which the profile being analyzed

Microparticles seem to be mainly distributed in the furrows filling the microrelieves. In this case the value Ra after microparticles application (Ra= 36 µm) decrease considerably compared to skin no treated (Ra = 65 µm), as shown in the Figure 8.
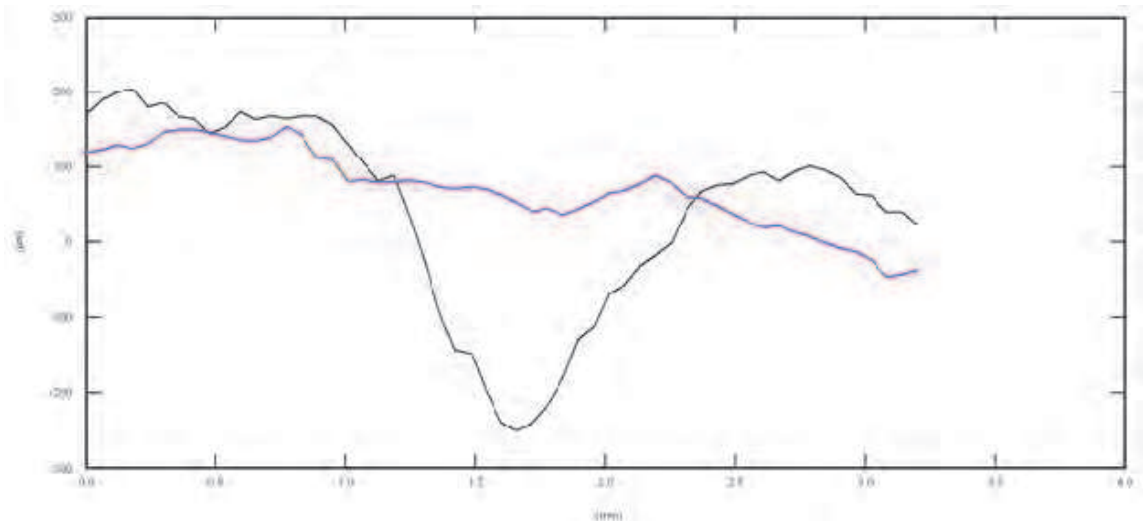
Fig. 8. Single profile of microrelief before (black curve) and after 1 minutes (blue curve) of microparticles application.

On the contrary, Ra value acquisition after nanoparticles application doesn't reveal a significant modification of the average roughness of microrelief (Ra before SLN application = 30 µm and Ra after SLN application = 38 µm) . This result could be probably due to the SLN nanosize that permits an homogeneous distribution both in relieves and in furrows supporting the film forming theory about the solid lipid nanoparticle interaction with the skin after cutaneous application (Figure 9).
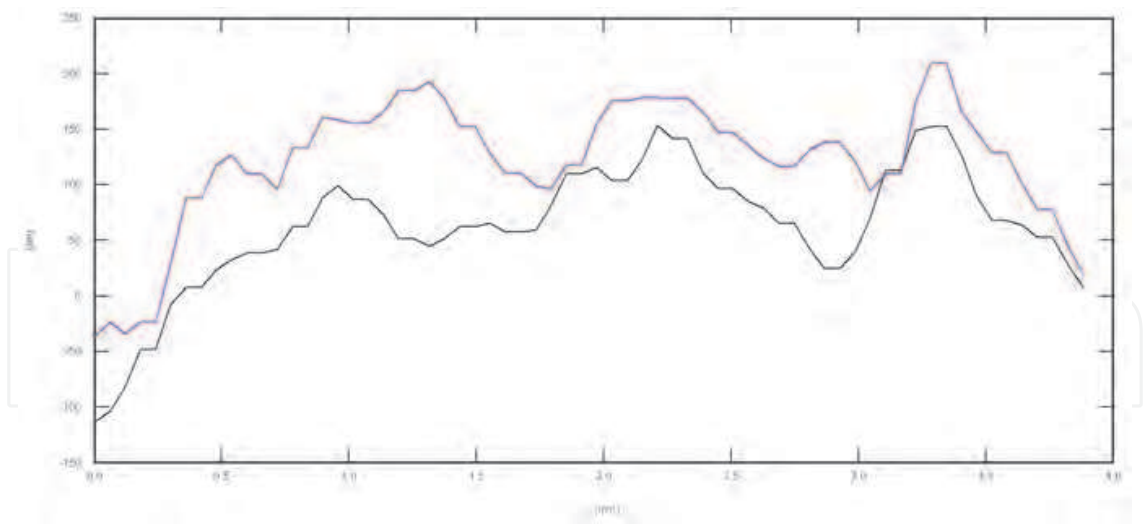
Fig. 9. Single profile of microrelief before (black curve) and after 1 minutes (blue curve) of solid lipid nanoparticle application.

The SLN form an adhesive film on the skin the furrows became less depth and the roughness parameters should reduce. Otherwise, penetration of the SLN in the stratum corneum through different routes should not lead to changes in roughness parameters. This hypothesis should be further investigated by combining this technique with other non-invasive methods of skin analysis.

4.3 High-frequency ultrasound and skin

The high-frequency scanners available today operate at frequencies between 20 MHz and 1-2 GHz. The optimal frequency range for dermatological questions is between 20 and 100 MHz [Gammal et al 1995].

Using a 20 MHz transducer it is possible to visualize structures up to 6-7 mm in depth. This means that the zones of diagnostic interest are covered i.e. epidermis, corium, and one portion of subcutaneous fatty tissue. Particularly, if the subcutis is not very well developed, evaluation of the muscle fasciae is also possible (Figure 10) .
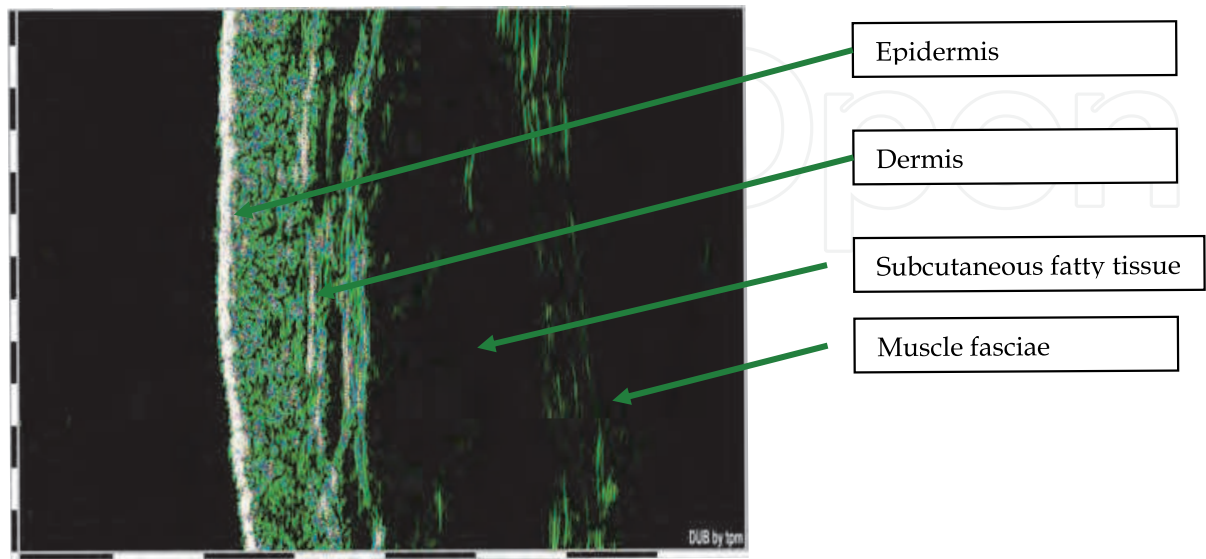

Fig. 10. Ultrasound B scan of a back skin of a 32 year old woman.

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Until now, no one has studied the interaction of nanoparticle systems with the skin by ultrasound scanning. Instead, the use of ultrasound may be helpful in investigating the effects of SLN application on the different layers of the skin.

4.4 Skin microcirculation

Skin microcirculation is rather complex. The skin viability depends upon the nutritional circulation, which cannot be assessed by conventional macrocirculatory methods that evaluate total blood supply. The indisputable advantage of the microcirculatory methods is to provide information directly in diseased skin areas and assess the effectiveness of vasoactive drugs where they are supposed to act.

Several techniques are available today to evaluate the skin microcirculation. Among them, capillaroscopy and transcutaneous measurement of the partial oxygen pressure are of special interest because they provide information which is directly useful in clinical practice [Li et al 2006].

The blood supply to the skin is provided by a network of arterioles, capillaries and venules organized into a superficial and a deep plexus. The assessment of skin microcirculation is of valuable interest in cosmetology in the quantification of the sun protection factor, skin irritation and efficacy of antiredness treatments. Skin microcirculation can be measured by means of different techniques, based mainly on the quantification of optical and thermal properties of the skin which are modified by the amount of blood perfusion. Relevant and reproducible data can be obtained only through the understanding of the biophysical background of the technique(s) utilized. Standardization of measuring conditions and procedures is particularly required for blood flow assessment. [Berardesca et al., 2002]

5. Conclusion

Micro and nano sytems are gaining great attention in biomedical applications such as design of drug carrier devices. Among them SLN have lots of advantages over conventional systems since they enhance the delivery, extend the bioactivity of the drug by protecting them from environmental effects in biological media, show minimal side effects, demonstrate high performance characteristics, and are more economical since minimum amount of expensive drugs are used.

After skin application of SLN three pathways of SLN penetration across the deeper layers of skin have been identified. That can to be exploited in order to promote the penetration of active ingredients into the dermis; in this way the active can to carry out a direct therapeutic and cosmetic action.

Potential systemic effects after dermal application of SLN should be considered in order to obtain a safe topical product. Skin penetration and systemic absorption should be estimated with the intention to assess the risk of using nanoparticles in topical products. Visualization of colloidal systems after skin application is essential to evaluate their interaction with cutaneous structures.

Non-invasive bioengineering techniques have become indispensable tools both in the development of drugs and cosmetics and in clinical dermatology. These techniques enable researchers to study the structure and function of human skin objectively and quantitatively.

These methods should help scientists working on formulations containing SLN in order to better understand the fate of nanoparticles after topical application and the effectiveness of their application to skin disorders and diseases.

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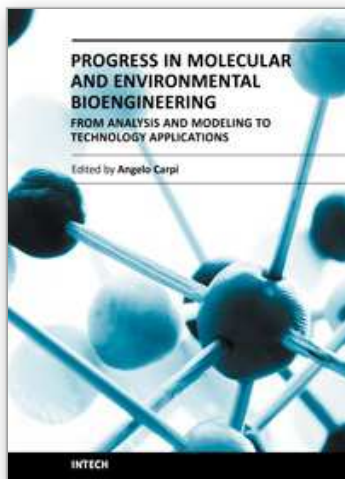
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