Mimicking skin: healthy and disease models for transdermal permeation studies

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Abstract

Skin drug delivery is an emerging route in the drug development, due to its great advantages, thus leading to an urgent need to understand the behaviour of active pharmaceutical ingredients into/through the skin. This knowledge is crucial in the early stages of product design and development. Yet, given the skin barrier properties as one of the first body's natural defence systems, it can act as an obstacle to the successful outcome of a skin drug therapy. To unravel the mechanisms underlying this barrier, reductionist strategies have designed several models with different levels of complexity and integrity, using non-biological and biological components. Besides the detail of information and resemblance to the in vivo Human skin that each in vitro model offers, the technical and economic efforts required should be considered when selecting the most adequate model for the intended research. This review provides an outline of the most commonly applied skin models, including healthy and diseased conditions, lab developed systems and commercialized models, their advantages and limitations and, also an overview of the new trends in skin engineered models.

Contents

Introduction

In the last decades, the study and development of mimetic skin models have been a hot topic of discussion mainly due to the rising of ethical questions and the establishment of new rules regarding the prohibition of animal testes. The need of new and efficient skin mimetic models remains crucial namely for pharmaceutical, cosmetic and toxicological purposes.

The safety and toxicity of new products and formulations must be assessed prior human application according to the European Union (EU) guidelines and to the Organisation for Economic Cooperation and Development (OECD) guidelines for testing dangerous ingredients for the skin (Kandarova et al., 2004, OECD, 2015, Fentem, 1999, Fentem and Botham, 2004, Worth et al., 1998).

Efficient methods for development and rationalization of drug formulations aiming topical application demand specific skin models capable of estimating the properties and the most suitable drug formulation. After the identification of important penetration and permeation properties (wanted and unwanted) for the drug formulations, its optimization becomes achievable (Flaten et al., 2015).

In line with this facts, several mimetic skin models have been developed and some are already available in the market, while the research continues for further improvements in their quality, complexity and mimetic properties. Different biomimetic materials have been used from the silicone membranes or poly(dimethylsiloxane)

(PDMS) models to human cell cultures as well as different technologies to simulate the highly complex and stratified structure of the human skin.

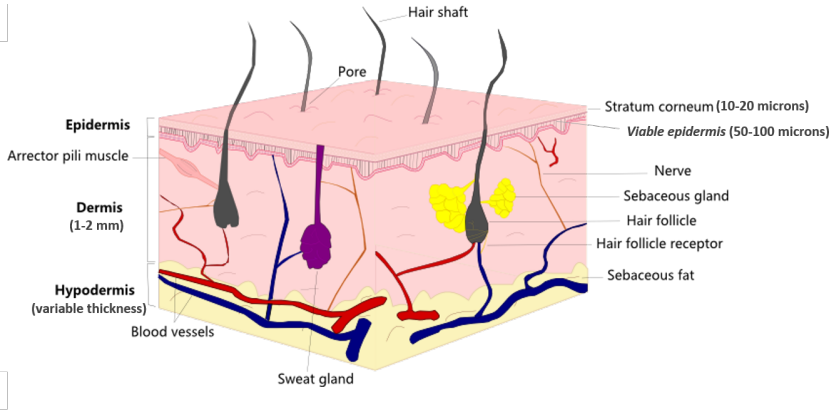
The big challenges in the development of skin substitutes are related with: a) the need of reproducibility in the results obtained using these models; b) their capacity to better mimic the multitude of human skin structure and functions and c) the development of cost-effective skin membrane models (Sarkiri et al., 2019, Flaten et al., 2015). The main focus of the present review is to summarize the currently reported healthy and disease skin mimetic models, discuss the characteristics and applications of these proposals and to identify the new trends in skin engineering.

Human skin – structure and functions

The skin is the major organ of the human body having a surface area of *at ca* 2 m^2, representing approximately 10% of the body mass for adults (Lee et al., 2006, Ng and Lau, 2015, Sofia A. Costa Lima., 2018). Skin is a physical barrier in the interface between the body and the external environment and constitutes a first-line defence entity for protection of the body, controlling what may enter and exit in the body. Moreover, skin is composed by a network of cells and matrix elements providing multifaceted functions such as the prevention of the body´s dehydration, protection of the body against infectious agents or ultraviolet radiation, thermoregulation, sensation and synthesis of vitamin D (Prausnitz et al., 2012, Sofia A. Costa Lima., 2018, Lee et al., 2006).

The organization of the skin consists of three major layers hypodermis, dermis and epidermis. The type of structure, cellular composition and major components of the three skin layers are summarized in Figure 1 (A and B), and these topics are discussed in more detail on the following subsections.

A



B

Layer	Structure	Cell types	Extracellular matrix components
Epidermis	Stratified keratinized epithelium	keratinocytes, melanocytes, Merkel cells and Langerhans cells	Keratin, type IV, VII collagen
Dermis	Blood vessels, nerves, mechanoreceptors, appendages	Fibroblasts, endothelial cells, Langerhans cells, muscle cells	Elastin, proteoglicans, type I, IV, VII collagen
Hypodermis	Blood vessels, nerves, hair follicles	Adipocytes, fibroblasts, endothelial cells, muscle cells	Elastin, type I collagen

Adapted from Navarro *et al*., 2019.

Figure 1 –Structure of human skin. A) Schematic representation; **B)** Characteristics of the three skin layers.

Epidermis

Epidermis constitutes the outermost barrier between the organism and the external environment and is itself organized in four layers the*stratum basale, stratum spinosum, stratum granulosum* and*stratum corneum* (*SC*) (Figure 1A). A complementary layer,*stratum lucidum* can be found on the palm and sole of the foot, which is responsible for the thickened skin. Nevertheless, the*stratum lucidum* is often considered the lower part of the*SC* but not an individual epidermal layer. There are also hair follicles and sweat ducts that cross different skin layers (Ng and Lau, 2015).

The most intrinsic layer of the epidermis, the *stratum basale* is composed by different types of cells: keratinocytes, melanocytes, Merkel cells and Langerhans cells, which participate in the immune response. The following layer, the *stratum spinosum* , is composed by keratinocytes. The third layer, *stratum granulosum* , consisting of several rows of keratinocytes and is a reservoir of ceramides which are relevant lipids for the constitution of the outermost layer, the*stratum corneum* (Sofia A. Costa Lima., 2018, Lee et al., 2006). Together, the three most intrinsic layers of the epidermis form the denominated "viable epidermis" (Ng and Lau, 2015). The viable epidermis is generally 50–100 µm thick and contains no blood vessels and sensory nerve endings (Ng and Lau, 2015, Prausnitz et al., 2012). Keratinocytes originate from the *stratum basale* and expose oneself to progressive differentiation while migrating to the *SC*they are necessary to maintain the *SC* by replacing lost corneocytes and *SC* lipids during desquamation (Ng and Lau, 2015). The viable epidermis is considerably hydrophilic ($> 50\%$) in the opposite to *SC* which is lipophilic and contains *at ca.* 10% of water (Forster et al., 2009).

Due to the importance of the *SC* for the permeation of drugs and the protection of the skin, the structure and functions of this layer will be more extensively discussed in this review in the following subsection.

Stratum corneum

The *stratum corneum* is typically 10-20 µm thick and is composed by 10–25 layers of dead mature keratinocytes, denominated as corneocytes, which are obtained from terminally differentiated keratinocytes during the passage from granular layers to *SC* . This process is characterised by loss of nuclei and cytoplasmic organelles, flattening and elongation of the keratinocytes. The continuous shedding of corneocytes from the skin surface is called desquamation (Ng and Lau, 2015) and it balances proliferating keratinocytes that constitute the*stratum basale* . The cells migration through the epidermis to the surface takes approximately fourteen days (Ovaere et al., 2009). Morphological dimension of the corneocytes are about 0.2 µm thick and 40–60 µm wide (Ng and Lau, 2015, Agache, 2004). Their membrane is 0.015 to 0.020 µm thick and includes involucrin and keratolinin as structural proteins (Pouillot et al., 2008).

The composition of *SC* is generally 20% of lipids and 70% of insoluble keratins (Walters and Roberts, 2002) and the organization of the layer is usually compared with the "bricks and mortar" model (Figure 2A). The bricks are the corneocytes and the extracellular matrix analogous is compared to the mortar in a brick wall, which is composed of lipids (stacked lipid bilayers that surround the corneocytes) like the cement holding the bricks together (Prausnitz et al., 2012, Uchida and Park, 2016, Menton and Eisen, 1971).

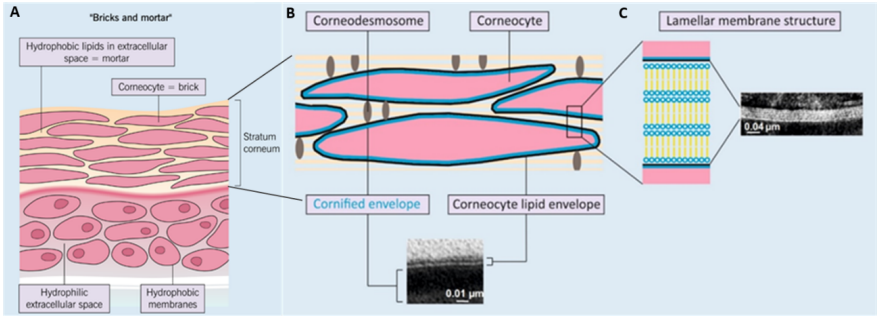


Figure 2 – *Stratum corneum* **structure and organization: A)** "Bricks and mortar" Two- model (adapted from (Prausnitz et al., 2012)); **B)** *Stratum corneum*structure: cornified envelope (blue); corneocyte lipid

envelope (CLE, black); corneodesmosome (brown). **C)**Lamellar membrane structure with intercellular lipids. Insert, electron micrograph: Murine skin was fixed in Karnovsky's fixative, and post-fixed with 1 % aqueous osmium tetroxide, containing 1.5 % potassium ferrocyanide (adapted from Uchida (Uchida and Park, 2016)).

The interior of the corneocytes is filled with keratin filaments embedded in a matrix mainly consisted of filaggrin (filament aggregating histidine-rich protein) and its breakdown products. These amino acids together with certain ions, such as chloride, sodium, lactate and urate form the natural moisturizing factors (Ovaere et al., 2009). These substances are endogenous humectants that keep the hydration of the *SC* on an adequate level. It is important for skin elasticity and permits regularly function for hydrolytic enzymes of desquamation (Fowler, 2012). Reduced levels of filaggrin and properly natural moisturizing factors are caused by mutations of the filaggrin gene connected with atopic dermatitis, ichthyosis vulgaris, psoriatic skin, ichthyosis and general xerosis (dry skin) (Uchida and Park, 2016, Fowler, 2012). The cornified envelope (CE) that encloses the Corneocytes is formed by structural proteins crosslinked by transglutaminases (Prausnitz et al., 2012). CE has a stable stiff property to withstand mechanical barrier stress (Figure 2B) and is surrounded by a layer of corneocyte lipid envelope (CLE) (Uchida and Park, 2016). This CLE is a support to form lamellar membrane structures and controls exit of hydrophilic agents from corneocytes, but the roles of CLE in the *SC* remain totally understood (Uchida and Park, 2016).

The extracellular space in the *SC* is filled by lipid-enriched layers (Uchida and Park, 2016) which are composed by ceramides (45–50%), cholesterol (25%), cholesterol sulphate (5%) and free fatty acids (FFAs) (10–15%) (Ng and Lau, 2015, Uchida and Park, 2016, Madison, 2003). The lipids form lamellar membranes that stretch out in a horizontal direction parallel to the corneocytes (Figure 2C). In addition, corneodesmosomes are found in the lamellar membrane structure. They are modified desmosomes including corneodesmosin, a structural protein that determines corneocyte adhesion (Ovaere et al., 2009). Corneodesmosomes consist of desmoglein-1 and desmocollin-1 connected to other corneocytes, intensify cohesion and regulate desquamation by their pH-dependent degradation (Uchida and Park, 2016).

Ceramides are mainly responsible for the skin renewal process (Mizutani et al., 2009) and play a critical role for the lamellar organization of this layer barrier (Prausnitz et al., 2012). At least 14 different classes of ceramides have been isolated from the human epidermis (Meckfessel and Brandt, 2014, Jurek et al., 2019, Sofia A. Costa Lima., 2018, Mojumdar et al., 2016). Each ceramide molecule contains a polar head group from sphingoid nature (sphingosine, phytosphingosine, 6-hydroxysphingosine or dihydrosphingosine) and two hydrocarbon chains derived from a fatty acid, a sphingoid or fatty acid ester moiety.

Cholesterol is the second most prevalent lipid by weight in the*SC* (Zbytovská et al., 2008, Pouillot et al., 2008, Prausnitz et al., 2012). The increment of cholesterol concentration in the lipid bilayers correlates with the diminution of the membrane thickness and density, and at the same time with an extension of the membrane surface area. Cholesterol fluidizes the *SC* lipid bilayers at skin temperature (Ng and Lau, 2015, Zbytovská et al., 2008).

The content of FFAs is 10 – 15% of the *SC* lipids and they consist mainly of very-long-chain species with [?]18 carbon atom and are mostly saturated, like ceramides.

In addition to lipid species, cholesterol sulphate is typically at 2–5 % weight ratio. Its potential functions are to help in the formation of the lipid lamellae and stabilization of the *SC* by inhibiting enzymatic degradation of corneodesmosomes (Prausnitz et al., 2012, Ng and Lau, 2015).

The majority of lipids is synthetized by keratinocytes in *stratum granulosum* and packed in lamellar bodies. These organelles deliver their lipid content in the extracellular space by fusion with the plasma membrane of keratinocytes of the *stratum granulosum* (Pouillot et al., 2008). Any changes in the concentrations of these main lipid components can damage barrier integrity that mediates normal barrier function.

These lipid structures prevent the excessive loss of water from the organism and block the entry of most topically administered drugs that have high molecular weight and low lipophilicity. Thereby, this barrier represents a great challenge for drug delivery across the skin, intended either for local effects or for systemic

therapy (Groen et al., 2008).

Concerning the functions of *SC* , it is established that this layer is responsible for the barrier and immune functions, namely preventing excessive water loss, maintaining body temperature, preventing the entry of xenotoxic chemicals and allergens as well as the invasion of microbes. Its functions also comprise the protection of the epidermis from oxidative stress and from mechanical stress.

The epidermal permeability barrier prevents intake and uptake of compounds. The *SC* barrier functions may be partially connected to its low hydration of 15%–20% and its very high density (1.4 g/cm^3 in the dry state) (Walters and Roberts, 2002). The body temperatures maintenance and water balance are regulated by blocking surplus evaporation of water from nucleated layers. One of the basic properties of this barrier is the prevention of penetration of allergens, microbial pathogens and xenotoxic chemicals (Uchida and Park, 2016). As a general rule, a compound with molecular weight larger than 500 Dalton cannot pass through the *SC* (Bos and Meinardi, 2000).

The epidermis has a pH gradient: the extracellular pH stays neutral between the *stratum granulosum* and *SC* , where it becomes more acidic to ca. 4.5 at the outer *SC* layer. Acidification enables antimicrobial activity and regulates, by enzymatic activity performed by proteases, the formation and desquamation of the epidermal barrier. The use of alkaline or neutral soaps lead to the increase of the *SC* pH, which conducts to untimely cleavage of corneodesmosomes, decline in *SC* cohesion and following impairment of the epidermal barrier. The pH changes are reported to matter in the pathogenesis of skin diseases such as atopic dermatitis, acne vulgaris and *Candida albicans* infections (Ovaere et al., 2009). For example, with pH below 5.5, the growth of *Pseudomonasacne* , *Staphylococcus epidermidis* and a problematic microbial pathogen, *Staphylococcus aureus* , are inhibited (Uchida and Park, 2016). The permanent bacterial flora in the skin presents a comprehensive ecosystem. *Staphylococcus* and *Micrococcus*strains and *Diphtheroid bacilli* are the main part of nonpathogenic microflora. They consume the sebum like basic nutrient and confine skin colonization by potentially pathogenic organisms (Pouillot et al., 2008).

SC plays an important role in the innate immunity, which is related with the presence of antimicrobial peptides such as cathelicidin, dermcidin, RNase7, S100A7/psoriasin and defensins (Uchida and Park, 2016). They have revealed potent antimicrobial activities against a wide spectrum of microbes, including gram-negative and gram-positive bacteria, fungi and some viruses. Cathelicidin antimicrobial peptide (CAMP) is inducible with infection, injury or inflammatory response and stimulates the production of a signal lipid, sphingosine-1-phosphate (S1P) under stress conditions and activate vitamin D receptor (VDR). Also, defensins are classified in three subfamilies, α-, β-, and ϑ-defensin. They are inducible peptides in epidermis in response to microbial infection, inflammation. and differentiation (Uchida and Park, 2016). Adaptive immunity in the*SC* is associated with the availability of urocanic acid. The*trans* isomer of urocanic acid is generated from histidine (principally from NMF) by histidase but can be converted to the*cis* isomer through exposure to ultraviolet (UV) radiation.*Cis* -urocanic acid binds to the serotonin [5-hydroxytryptamine (5-HT)] receptor to eliminate immune function (Egawa et al., 2010). Urocanic acid is an epidermal major chromophore, which works as a powerful endogenic UV absorbent. Most lower UV wavelengths (UVB = 280–315 nm) are absorbed in the epidermis, but longer wavelengths (UVA = 315–340 nm) get to the dermis. The bulk amounts of proteins, lipids, and nucleotides has individually low potent chromophores, but they can form the UV barrier together (Uchida and Park, 2016). The *SC* is constantly exposed to oxidants, including UV light, chemical oxidants and air pollutants. α- and γ-Tocopherol, ascorbic acid and glutathione are the chief hydrophobic antioxidants of *SC* , providing the lipid bilayers stability and safeguarding from lipid peroxidation (Pouillot et al., 2008).

Dermis

The dermis is the bigger layer of the skin, with a thickness of approximately 1–2 mm and provides important physical properties, namely flexibility, elasticity and tensile strength. It is an integrated system of fibrous and connective tissue, composed by collagenous and elastin fibbers, accommodating epidermally derived appendages (hair follicles, nails, sebaceous glands and sweat glands) and sensory nerve endings, lymphatic

vessels and blood capillaries. Thus, metabolic exchanges between the epidermis and the blood systems may occur as well as clearance of cell metabolic products and penetrated foreign agents. The dermis contains resident cells (e.g., fibroblasts and mast cells) and cells from the immune system, including macrophages and dermal dendritic cells.

The thermal barrier, energy depositary and protection from physical stroke are mainly connected with adipose tissue associated with collagen fibbers, found in the lower reticular dermis layer. The water content reaches 70%, favouring hydrophilic drug uptake. Below the reticular dermis layer, the fibrous connective tissue transitions to the adipose tissue of the hypodermis. This is mainly constituted of adipocytes interconnected by collagen fibbers, forming a thermal barrier able to store energy and protect from physical shock (Sofia A. Costa Lima., 2018, Forster et al., 2009).

Hypodermis

The subcutaneous layer or hypodermis is the innermost layer of the skin and consists of fat cells. However, this layer can be absent in some thin skin, for example on the eyelid. The hypodermis is between the skin and the subjacent tissues of the body, such as muscles and bones. Larger lymphatic and blood vessels are standing in this layer. Consequently, the major functions of the hypodermis are insulation, mechanical integrity and support and conductance of the vascular and neural signals of the skin (Alkilani et al., 2015, Ng and Lau, 2015).

Drug skin penetration routes

The skin is an attractive site for delivery of drugs and cosmetics. But normal skin is a serious barrier to drug absorption, which is why pharmacologists and cosmetologists became interested in the development of new drugs, formulations and ways of delivery.

Drugs can be administrated through the skin providing a local action (topical administration) or a systemic effect, reaching the bloodstream. The types of administration of drugs through the skin can be organized in many ways, depending of the criteria selected. A possible classification can be performed considering the invasive and non-invasive nature of the different routes. Regarding invasive routes of administration, they can be categorized as the routes in which the drugs can enter through the skin by needle injections (subcutaneous, intramuscular or intravenous routes) and those that consist in the implantation of a device. In the subcutaneous route, the needle is inserted into fatty tissue thus reaching the bloodstream. This type of administration is usually used for the administration of many protein drugs (eg. insulin) as they are destroyed in the digestive tract. Intramuscular route is considered preferably over subcutaneous route for the administration of larger volumes of drugs. In the intravenous route, the drug is administrated directly into a vein and is used to give a drug in a rapid and in a well-controlled manner the drug is delivered immediately to the bloodstream. Drugs can also be administrated by implantation of a device which is inserted under the skin. This type of administration is probably the less commonly used and is usually considered for the delivery of the drugs by controlled release along the time for longer periods .

Considering the non-invasive methods, there are four possible routes of drug penetration across the skin: intercellular, intrafollicular, transcellular and polar (Pouillot et al., 2008), as depicted in Figure 3A. Sometimes, the diffusion through the skin appendages (e.g., fair follicles, sebaceous glands and sweat glands) is classified as appendageal route (Prausnitz et al., 2004, Ng and Lau, 2015). Intercellular and transcellular ways are considered transepidermal pathways.

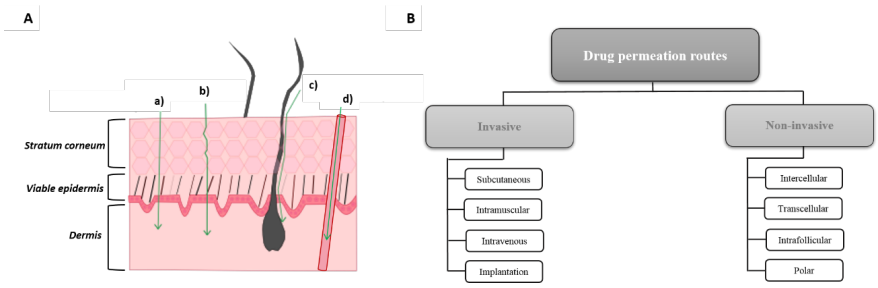


Figure 3 – Skin Drug Delivery. A) Pathways into the skin for transdermal drug delivery: a) Transcellular pathway (penetration through the corneocytes); b) Intercellular pathway (penetration between the corneocytes through the intercellular lipids); c) Intrafollicular pathway (penetration through the hair follicles); d) Polar pathway (penetration through the polar pores); adapted from (Sofia A. Costa Lima., 2018); **B)** Types of drugs entrance routes through the skin.

The intercellular pathway involves the passage of the drugs through the lipid matrix that occupies the intercellular spaces of the corneocytes and is usually the preferred route for lipophilic substances. Otherwise, the transcellular pathway, also known as the intracellular pathway, occurs through the successive skin layers and dead cells and allows the transport of hydrophilic or polar substances. The transappendageal pathway uses the different skin appendages to enter through the skin. Various sweat glands, hair follicles and pores opening to the outer surface of the skin via their ducts can be used as a possible way for the entrance of drugs. These polar pores are located between cells and encircled by polar lipids, which make small holes in *SC* (Alkilani et al., 2015, Sofia A. Costa Lima., 2018). Hence, it was considered an inessential pathway for drug penetration but nowadays, current research suggests that hair follicles and sweat glands may present an alternate pathway for a diffusing molecule (Uchechi et al., 2014). In the polar pathway, the penetration of the drugs occurs through the polar pores available in the skin.

When the drugs are able to penetrate deep in the skin, from the surface through the various layers, this type of penetration is called "transdermal drug delivery" (TDD). The drug firstly pass through the*SC* and then permeates via the viable epidermis and dermis by diffusion. After reaching the dermal layer, the drug becomes available for the uptake into the systemic circulation (Alkilani et al., 2015). TDD has advantages namely over hypodermic injections as the drugs (usually administrated in patches) can be applied only one time and released for a longer period of time (with no need of additional applications), is almost pain-free and doesn't lead to the generation of dangerous medical waste such as needles and syringes. Furthermore, transdermal devices can be self-administered, and the administration can be easily stopped in case of need by removal of the patch (Van Gele et al., 2011).

The different types of drugs entrance routes through the skin and their classification are summarized in Figure 3B.

Healthy skin mimetic models

Despite de fact that the *in vivo* human skin is the most realistic and gold standard experimental model for the investigation of drugs interaction with the skin, the use of this model is not always possible mainly because of the ethical concerns, regulatory issues, laboratory facilities and the potential risk associated to the eventual toxic effects of the drugs (Van Gele et al., 2011). Moreover, the results obtained by the use of human *ex vivo* models present significant variability because samples are usually obtained from different anatomical places of the same donor, different donors and have unpredictable character depending on the different subjects or different age groups (Flaten et al., 2015). These facts reinforce the need of alternative models able to better mimic the real scenario of drug interaction with the skin and concomitantly allowing reproducible results (Abd et al., 2016).

In this section, an overview of the existent *ex vivo* and *in vitro* mimetic skin models will be given.

Ex vivo **human and animal models**

During long time, the main way for the preclinical research of new drugs and for the optimization of topical drug formulations was the investigation considering the use of *ex vivo* skin mimetic models. The literatures describe two main groups of *ex vivo* models obtained from human or animal organisms (see references (Abd et al., 2016, Flaten et al., 2015) for reviews).

Human skin is absolutely the most suitable model for study TDD (Ruela et al., 2016). The skin samples used in *ex vivo* permeation assays can be obtained from different origins namely from plastic surgeries, amputations or cadavers and in generally the skin excerpts can be collected from different organs, such as the abdomen, back, leg or breast (Schaefer et al., 2008). Different membrane types can be obtained by using human skin excerpts for further use in drug permeation studies. Full-thickness skin models, in which the excisions containing connective tissue and subcutaneous fat and consists of all layers below, including the dermis, are reported as useful model to test different drugs and formulations (Abd et al., 2016, Cross et al., 2003, Manca et al., 2014, Junyaprasert et al., 2012, Dragicevic-Curic et al., 2008, Dragicevic-Curic et al., 2010, Elmoslemany et al., 2012, Bragagni et al., 2012, Cal, 2006, Sahle et al., 2014, Gaur et al., 2013, Marimuthu et al., 2012).

Ex vivo epidermal membranes models are also used for permeation experiments and those models are obtained from thermal treatment of full-thickness skin (immersion in hot water) (Junyaprasert et al., 2012, Kligman and Christophers, 1963) or by chemical action namely by the use of different reagents such as ethylenediaminetetraacetic acid, ammonia and enzymes (Cross et al., 2003) in order to separate the membrane at the dermal–epidermal junction. Other methodologies using human dermatomed skin (Dragicevic-Curic et al., 2010, Dubey et al., 2007, Clares et al., 2014, Marepally et al., 2013) or dermatopharmacokinetic method in which tape stripping is used to remove *SC* layers have been described (reviewed in (Abd et al., 2016)).

More recently, abdomen skin samples from patients who underwent abdominoplasty are used as skin models (Ternullo et al., 2018). Many examples report the use of human *ex vivo* skin models (reviewed in references (Flaten et al., 2015, Abd et al., 2016)), as the investigation of the dermal uptake and percutaneous penetration of some organophosphate esters in a human skin *ex vivo* model (Frederiksen et al., 2018). In another study, the effect of some nanoemulsions containing alpha-tocopherol was evaluated in skin wounds either in cell lines and using *ex vivo* human biopsies samples (Bonferoni et al., 2018).

The use of skin perfusion models, a surgically prepared portion of skin including subcutaneous fatty tissue with assured continuous vascular circulation is reported (Ternullo et al., 2017a, Ternullo et al., 2017b), to test different drugs, namely nanoparticle formulations. The use of this model is considered a promising strategy since they present benefits over *in vitro* models, as they overcome the existence of only epidermis and part of the dermis and the lack of a vascular system as verified in the most commonly used *in vitro* models (Ternullo et al., 2017a, Ternullo et al., 2017b).

Regarding animal *ex vivo* models, pig skin models are the most relevant because of the multiple anatomical, physiological and histological similarities with the human skin such as the dermal/ epidermal thickness ratio, epidermal thickness, similarity in hair follicle and blood vessel density in the skin and content of *SC*ceramides, dermal collagen and elastin (Abd et al., 2016). The pig skin is easily obtained as a waste from animals slaughtered for food. Amongst the different parts of the pig body, the central outside part of the porcine ear has been the mostly recommended due to the analogy with human skin layers (Meyer et al., 2006). Variability of permeability in different samples of pig skin also takes place. The pig ear skin permeability is comparable with human skin. In fact, studies showed a good correlation especially for lipophilic substances. Furthermore, the age of the animal influences the permeability of the drugs, however most of literature does not specify the age of animal (reviewed in (Flaten et al., 2015)).

Many different drugs and formulations such as liposomes, nanoparticles and microemulsions have been studied using *ex vivo* pig skin models. Amongst the number of studies available, some reports describe the evaluation of the permeation of liposomes containing different drugs in excised pig ear (Scognamiglio et al., 2013, Knudsen et al., 2012, Gillet et al., 2011). Other studies tested the permeation of different nanoparticles

in pig ear models (Gomes et al., 2014, Pople and Singh, 2011, Şenyiğit et al., 2010). Most recently, new formulations including a transferosomal gel were tested using pig ear skin as an *ex vivo*model for the study of the transdermal permeation and delivery of the drug (Das et al., 2017). The use of excerpts from other pig skin regions, namely from abdomen (Nagelreiter et al., 2013) and dorsum (Hathout et al., 2010) is also described. Furthermore, newborn pig skin excisions are used as skin models for evaluation of topical drug formulations (Cilurzo et al., 2007).

In addition to the pig skin models, several other animals are used namely primates, mice, rats, guinea pigs, rabbits, bovines (udder) and snakes (shed skin). However, these models require ethical permissions. Since 2009, the use of animals for collection of toxicological data for cosmetic ingredients has been prohibited in the EU (76/768/EEC, February 2003) (Van Gele et al., 2011).

Mainly due to the fact that primate research is highly restricted and very expensive, skin of rodents (mice, rat and guinea pigs) is sometimes considered for permeation studies, due to its high availability, small size and quite low price. There are available many hairless strains which can be advantageous for this type of studies (Abd et al., 2016). Amongst rodents, rat skin is most like human skin however many studies pointed out the fact that its skin is more permeable than human skin (Barber et al., 1992, Chowhan and Pritchard, 1978, Hughes and Edwards, 2010, Schmook et al., 2001, van Ravenzwaay and Leibold, 2004). Yet, a study has shown that hairless mouse skin is an inadequate model for assessing the effects of the skin penetration enhancers (Bond and Barry, 1988).

Shed snake skin was considering as well as a useful model to mimic human skin and it can be obtained without killing the animal however it lacks hair follicles (Rigg and Barry, 1990, Itoh et al., 1990, Wonglertnirant et al., 2012, Kumpugdee-Vollrath et al., 2013). Additionally, udders from slaughtered cows are used as an *ex vivo* animal model and studies involving the comparison of this model with porcine skin has confirmed that both models are well correlated, thus enabling its use for studies regarding topical administration of drugs (Netzlaff et al., 2006).

The several *ex vivo* animal models mainly differ in the thickness of *SC* , hair density, number of corneocyte layers, hydration, lipid profile and morphology which may constitutes several advantages and limitations of each model. The most relevant features are summarized in Table 1 (Flaten et al., 2015).

Table 1 – Characteristic of *ex vivo* skin models in relation to human skin.

Ex vivo Skin model	Advantages	Disadvantage
Human excerpts	Gold-standard model	Ethical permission High inter- and intra-variability than with porcine ear skin Storage stability lack
Pig (ear)	No ethical restrictions Availability (waste from slaughter) Similarity with human skin	Age of animal influences skin thickness Hair follicles (removal may conduce to skin damage) Storage stability lack
Newborn pig	Similar thickness of the *SC*	Higher number of hairs than in humans Variability due to the use of different anatomical sites Storage stability lack
Mouse / Rat	Small size Uncomplicated handling Hairless species available	Ethical restrictions Lower thickness High permeability High density of hair follicles Removal of hairs (skin damage)
Guinea pig	Similar permeability to human skin Hairless species available	Ethical restrictions High density of hair follicles Removal of (skin damage)

Ex vivo Skin model	Advantages	Disadvantage
Rabbit	Ears as waste from slaughter Similar permeability to guinea pig	Ethical restrictions High density of hair follicles Hair follicles (removal may conduce to skin damage)
Shed snake	Single animal provides repeated sheds Multiple samples from one animal Storage at room temperature	Absence of hair follicles Different skin metabolism Absence of living epidermis and dermis
Bovine udders	Availability (easily obtained waste from slaughter) Multiple samples from one animal	Weaker barrier to some drugs than pig skin Storage stability lack

Adapted from (Flaten et al., 2015).

In vitro **membrane models**

In order to overcome the disadvantages of *ex vivo* human and animal models, mainly related with the availability and the ethical problems, numerous efforts have been done to develop alternative skin equivalents based on *in vitro* approaches. *In vitro* skin alternative models may consist either on mimetic membranes (non-lipid and lipid systems) or cell cultures (Abd et al., 2016, Flaten et al., 2015, Berben et al., 2018, Sinkó et al., 2014, Faller, 2008). The existent models and their applications will be reviewed in the following sections.

The first studies regarding the development of *in vitro* skin equivalents developed in mid 70s included the use of normal human keratinocytes (NHKs) as a model for skin irritancy (Rheinwald, 1989). The cell culture of NHKs begins with a small piece of human skin (about 0.5-1 cm^2) obtained from surgery after separation and specific treatment, that grows easily in culture medium. It allows the use of a large number of cells, leading to the opportunity for wide ranging toxicity screening tests with many substances. This model shows good ability for testing hydrophilic compounds however presenting less capability for the evaluation of poorly water soluble compounds and complex formulations (reviewed in (Ponec, 1992)).

The model was improved with its application in membranes, which support the NHKs during growing and forming the called reconstructed human epidermis. More complex models mimicking the full thickness skin consisting of the fibroblast populated collagen matrices (dermis equivalent) and an epidermal cover composed by NHKs (Van Gele et al., 2011).

Nowadays there are different commercially available models of the human epidermis or full thickness skin. These models will be reviewed with more detail in section 3.2.3.

Additionally, other acellular *in vitro* models have been described: a) non-lipid-based models like the silicone membranes or poly(dimethylsiloxane) (PDMS) models (Oliveira et al., 2011) and b) lipid-based models like Parallel Artificial Membrane Permeability Assay (PAMPA) (Sinko et al., 2012, Sinkó et al., 2014) and Phospholipid Vesicle-based Permeation Assay (PVPA) systems, as simpler alternative approaches (Engesland et al., 2013).

Non-lipid-based models

The first studies regarding the use of non-lipid-based skin models date from 1970 and report the use of silicone membranes to study the release of salicylic acid (Nakano and Patel, 1970). Later, some studies reported the use of different microporous membranes based on pure cellulose acetate, cellulose and polysulfone to investigate the permeation of hydrocortisone from two commercial creams (Shah et al., 1989). Other synthetic membranes considering polysulfone, cellulose mixed esters, polytetrafluoroethylene and polypropylene in their composition were described and used to study the nitroglycerin release from commercial ointments

(Wu et al., 1992). More recently, some studies describe the interaction of many drugs and vehicles with skin models of PDMS or with silicone membranes skin mimetic models (Dias et al., 2007, Watkinson et al., 2009a, Watkinson et al., 2009b, Watkinson et al., 2011, Oliveira et al., 2011, Oliveira et al., 2010, Oliveira et al., 2012).

These skin equivalents are simple models with great applicability to test a basic diffusion mechanisms however they present some disadvantages namely the lack of similarity with the human skin and they are not much useful in the study of the permeability of hydrophilic compounds despite the good results obtained for lipophilic drugs (Abd et al., 2016, Miki et al., 2015).

Another study reports a new and improved model based on a membrane impregnated with a polymer of PDMS and PEG 6000 and some preliminary results elucidating the permeation of drugs in the aqueous solutions were described by Miki and co-workers (Miki et al., 2015).

Lipid-based models

Parallel Artificial Membrane Permeability Assay

The lipid-based skin equivalents appeared as valuable alternatives to non-lipid-based models, improving its complexity and the ability to mimic the human skin, namely the *SC* layer.

A first report dates from 1989 and describes the preparation of model membranes, using *SC* lipids, to study the permeability of drugs in the skin. The authors consider ceramides, cholesterol, FFA and cholesteryl sulphate in its composition (Abraham and Downing, 1989). The selected lipid composition agrees to those found in the human *SC*layer and thus it constitutes an advantage for this model since it better represents the layer in terms of its lipid composition. However, the most consistent studies started in 1998 when Kansy and co-workers reported the first PAMPA model as a tool for rapid determination of passive membrane permeability of drugs (Kansy et al., 1998). This system included a mimetic membrane from hydrophobic filter coated with phosphatidylcholine dissolved in n -dodecane as a membrane barrier, which differentiates the donor and acceptor parts. Even though this model was oriented for testing the transcellular intestinal permeability, it was the precursor of the following models, especially those developed to mimic the human skin.

The first PAMPA model for skin penetration estimation was published by Ottaviani and co-workers in 2006 (Ottaviani et al., 2006) however this proposal is, in fact, a non-lipid-based model which incorporates silicone oil and isopropyl myristate as membrane components. This model was used to study the permeation of a large variety of drugs and vehicles (Dobričić et al., 2014, Markovic et al., 2012, Karadzovska and Riviere, 2013, Karadzovska et al., 2013).

In the same year, Loftsson and co-workers described a novel skin substitute membrane based on a hydrated semi-permeable cellophane membrane and a lipophilic membrane of pure n -octanol in a nitrocellulose matrix. This model was used to study the permeation of different cyclodextrin formulations and the results have shown that the drug permeation patterns of the different formulations were similar to those previously observed for biological membranes (Loftsson et al., 2006).

In 2012, Sinko *et al* . designed the skin-PAMPA (Sinko et al., 2012), which consist of ceramides analogues called the synthetic ceramides or certramides, as substitution for the native ceramides found in *SC* . Certramides are cheaper alternatives to natural ceramides with the potential to prolong the storage time. This skin-PAMPA model was used in the skin permeability studies and exhibited poor correlation with skin epidermis, however it presented a good correlation with full thickness skin (Sinko et al., 2012). Even though its composition intents to simulate mainly the *SC* , in some cases, skin-PAMPA can even be a valuable alternative to replace models that use real human skin. Moreover, it constitutes an easy, quick and cost-effective research tool to study the permeation of pharmaceutical and cosmetic ingredients.

More recently, a modified version of the skin-PAMPA was reported (Tsinman and Sinkó, 2013) and used to evaluate the skin permeation of different some ibuprofen-containing formulations. The developed system was

able to distinguish between the different types of formulations the results correlate well with those found in permeation studies using human epidermis as a mimetic system.

Several other reports can be found in the literature reporting the use of PAMPA models to investigate drugs' permeability in the skin (Markovic et al., 2012, Dobričić et al., 2014, Faller, 2008, Köllmer et al., 2019, Alvarez-Figueroa et al., 2011, Vizserálek et al., 2015, Wu et al., 2019, Luo et al., 2016, Zhang et al., 2019, Balázs et al., 2016). The vast number of reports considering the use of this type of mimetic models to study the interaction and permeation of many different bioactive ingredients highlights the large spectra of applicability of PAMPA approaches and points out the ability of these barriers to predict the permeation of drugs in the human skin, despite of the more simplistic composition of these systems.

Phospholipid Vesicle-based Permeation Assay

The original PVPA ($PVPA_o$) was introduced as a model for screening the intestinal permeability and is composed of a consistent coat of liposomes deposited on a filter support acting as biological barrier (Flaten et al., 2006b, Flaten et al., 2006a). Later, by changing the lipid composition of the liposomes used to produce the permeation barrier, a new PVPA model was developed aiming to mimic the *SC*barrier of the skin (Engesland et al., 2013). In this model, liposomes are located within the pores and on the surface of the membranes (Flaten et al., 2006a). Thereafter, other modified versions have been reported (Berben et al., 2018, Engesland et al., 2013, Engesland et al., 2015, Engesland et al., 2016, Ma et al., 2017, Palac et al., 2014, Zhang et al., 2016).

Mainly, two skin PVPA models, presenting different lipid composition, for estimating skin penetration have been described: a) PVPAc - liposomes made of egg phosphatidylcholine (EPC) (77.1%, w/w) and cholesterol (22.9%, w/w) (Engesland et al., 2013) and b) PVPAs - lipid mixture of EPC (50%, w/w), ceramide (27.5%, w/w), cholesterol (12.5%, w/w), FFA (7.5%, w/w) and cholesteryl sulfate (2.5%, w/w) (Engesland et al., 2013).

The permeability of different compounds was evaluated in these PVPA skin models and the results were compared with reported permeabilities using animal skin models (rat, cattle, dog and pig) and with estimated*in silico* values. The PVPA permeability data mainly corresponded with the literature values of the animal skin penetration assays and the*in silico* values, with the exception of flufenamic acid that showed a relatively lower permeation (Engesland et al., 2013).

Later, the PVPAs model was examined with diverse formulations made of different types of liposomes containing diclofenac sodium salt. The results showed a rising permeation ranking of diclofenac sodium from liposomal formulations correlating with the physicochemical parameters of the liposomal vehicle. The permeability of diclofenac increased in the availability of the penetration enhancers (Palac et al., 2014). The PVPA model was further optimized considering a complex skin PVPA containing all the classes of lipids found in the *SC* , and the penetration enhancing effect of menthol was investigated for a set of active compounds with different physicochemical properties (Ma et al., 2017).

The previously described PVPA skin models were studied in comparison with a reconstructed human skin model (EpiSkin®). The permeability results indicate that the PVPA model has the ability to distinguish between the liposomal formulations and drug solutions, as opposed to EpiSkin®. PVPA models were better than EpiSkin® concerning their potential to determinate the influence of the formulations on the drug permeability which could be used in drug development at early stage. Moreover, PVPA barriers revealed straightforward, effectiveness, economical and long storable properties (Engesland et al., 2015).

More recently, other two lipid-based models have been reported as skin mimetic systems and they were used for the study of the effect of a set of synthetic surfactants on the skin. The new models proposed contain DPPC and cholesterol in a molar ratio of 7:3 or a mixture of ceramide, stearic acid and cholesterol in a molar ratio of 14:14:10 (Jurek et al., 2019).

Recently, our research group recently developed and characterized a cheaper and simpler alternative *SC* mimetic model (Shakel et al., 2019) that simulates this human skin layer and can allow the screening of drug

candidates. The design of this new model was inspired on PVPA approaches and comprises a lipid composition which closer resembles that found in the human *SC* layer, namely in the percentage of ceramides considered in its constitution. Thus, the novel human SC PVPA model is made of liposomes composed by ceramide (50% w/w), EPC (25% w/w), cholesterol (12.5% w/w), free fatty acids (10% w/w) and cholesteryl sulphate (2.5% w/w). This model presents some advantages since it can be stored up to 2 weeks at -20 °C, without losing their integrity.

Cell-based skin models

The complexity of the skin mimetic models has been increasing along the time, starting from *in vitro* non-lipid-based to lipid models and later to models comprising simple or more complex cell cultures. Single or multi-cell type 2D cultures, in which cells are grown as a monolayer disposed on solid flat surfaces, such as polystyrene or glass, are some of the mostly used cell-based approaches due to their relative simplicity and cost-effectiveness (reviewed in (Randall et al., 2018)).

The first studies regarding 2D skin cell mimetics were performed by Rheinwald and Green and consist in the growing of human keratinocytes monolayer culture (obtained from foreskin of newborns) deposited on plastic culture plates (Rheinwald and Green, 1977). As a further advance, the authors included a primary fibroblast cell line (3T3) in the keratinocyte cultures in order to close resemble the skin. Furthermore, fibroblasts are important for the growth of keratinocyte cultures, namely by the secretion of extracellular matrix components, like collagen (Rheinwald and Green, 1975).

The successfully use of 2D skin mimetic models has been reported in many studies for skin irritation and drug development, as referred in (Ponec, 1992, Amelian et al., 2017). Silva and co-workers established an*in vitro* epidermal monolayer using human keratinocytes (HaCaT cells) differentiated in a high calcium concentration medium, and applied it in the evaluation of drug delivery systems, namely lipid-based nanocarriers (Silva et al., 2017).

Despite the good performance of 2D skin mimetic models, 3D cellular models represent more precisely the human skin due to complexity and stiffness. Bioengineered skin can be obtained by the building of reconstructed skin models which constitutes artificially fabricated skin mimetics. 3D (single or multi-cell type) reconstructed skin models consist in the deposition of different layers of human cells in culture on a polymeric matrix, thus conducing to the production of a more complex and interactive system. In some recent studies, the incorporation of melanocytes (Min et al., 2018, Dai et al., 2018a), adipocytes (Klar et al., 2017) and endothelial cells (Dai et al., 2018b) in 3D skin mimetic models has already been reported.

The main differences between 2D and 3D skin models and the advantages of each class of models are depicted in Table 2.

Table 2 – Main characteristics of 2D and 3D cell-based skin mimetic models.

	2D cultures	3D cultures
Structure	Single or co-cultured monolayers	Multi-layered cultures / stratification
Application	Initial studies on drug-cell interaction	Studies on complex drug-tissue interactions
Air-liquid interface	No	Yes, required for stratification
Preparation/ cultivation time	Days	Weeks
Costs	Low	High

Adapted from (Löwa et al., 2018).

According to the type of layer from the human skin that each model can mimic, two main types of reconstructed skin models are described: reconstructed human epidermis (RHE) models which mimic the epidermis and the living skin equivalents (LSEs) which aim to simulate the full human skin (Kuchler et al., 2013, Flaten et al., 2015, Randall et al., 2018). Nowadays, there are some commercially available models of the human

epidermis (EpiSkin®, EpiDerm®, SkinEthic®) or full thickness skin (Phenion®) (Van Gele et al., 2011, Netzlaff et al., 2005).

The construction of the more complex 3D models usually include fibroblasts cultures which are embedded in a 3D matrix to mimic the dermis layer; then, keratinocytes can be seeded on top of the latter layer to simulate the epidermis. An air-liquid interface is established in order to allow the differentiation keratinocytes and formation of the different epidermal sublayers. The artificial matrix may include in their composition collagen or fibrin fibbers or even alginate and chitosan or different synthetic polymers (reviewed in (Sarkiri et al., 2019, Yu et al., 2019)).

Skin engineered substitutes may be used not only as alternatives to *ex vivo* and *in vitro* non-cell-based models for testing drugs in healthy or pathological conditions but they can also be applied in patients for regeneration of damaged skin (reviewed in (Yu et al., 2019)). A diagram summarizing the different applications of skin engineered substitutes is depicted in Figure 4.

Hosted file

`image4.emf` available at https://authorea.com/users/304989/articles/435563-mimicking-skin-healthy-and-disease-models-for-transdermal-permeation-studies

Figure 4 - Diagram of the different applications of skin engineered substitutes. Adapted from (Sarkiri et al., 2019).

3D skin equivalents have been described in the literature such as a model composed by fibroblasts and keratinocytes grafted in a viscose rayon support, which was created to test potential skin irritants (Canton et al., 2010). Other model comprises the use of sucrose co-polymers and fibroblasts, thus leading to the formation of a macromolecular assembling which potentiate collagen deposition (Au - Benny et al., 2016). Uchino and co-workers developed a 3D human skin model containing vitrified collagen that supported the culture of dendritic cells, keratinocytes and fibroblasts in a layered construct (Uchino et al., 2009). Other three-layered constructs featuring a hypodermis-like layer have been reported as full thickness *in vitro* models of human skin (Trottier et al., 2008, Monfort et al., 2013).

Some reconstructed skin models are produced in the laboratory, for particular research purposes, however other models are already commercially available. Amongst these, there are different classes of systems, namely RHEs (eg, EpiSkin®, SkinEthic®, and EpiDerm®) and LSEs (eg, GraftSkin®, EpiDermFT®, and Pheninon®) models (reviewed in (Abd et al., 2016, Yun et al., 2018). Some of these models have been validated according to European Union (EU) guidelines and implemented into the EU and Organisation for Economic Cooperation and Development (OECD) guidelines for testing dangerous ingredients for the skin (Kandarova et al., 2004, OECD, 2015, Fentem, 1999, Fentem and Botham, 2004, Worth et al., 1998).

SkinEthic®, EpiDerm® and EpiSkin® are probably the most used models and their use was approved by the European Union Reference Laboratory for alternatives to animal testing (EURL – ECVAM) (OECD, 2011). The SkinEthic® and EpiDerm® consist of epidermal keratinocytes cultured on polycarbonate membrane whereas EpiSkin® is composed by stratified human keratinocytes cultured on collagen-based matrix (Yun et al., 2018).

Schäfer-Korting and co-workers have extensively reported a comparison of the permeation of several hydrophilic and lipophilic compounds in human epidermal membranes, porcine skin and three RHE models (SkinEthic®, EpiDerm® and EpiSkin®). The results pointed out that the RHE models, mostly SkinEthic®, were significantly more permeable than human epidermis and pig skin, however the permeation of the compounds through pig skin and the RHEs is similar to those obtained in human epidermis. Interestingly, they did not observe the expected improvement in reproducibility with the RHEs compared to the *ex vivo* skin (Schafer-Korting et al., 2008).

Other 3D models were designed and commercialized to mimic the *SC* , like Strat-M®, however, this model is not a cell-based system since it is absent of cells. Strat-M® is a synthetic membrane comprising multiple

layers of different types of materials, as porous polyether sulfone and polyolefin, enclosed by a combination of lipids (ceramides, cholesterol, free fatty acids) and other components. Strat-M® was used to evaluate the permeation efficiency of hydrophilic molecules and to study the mechanism of passive transport (Uchida et al., 2015, Haq et al., 2018). Even though this model lacks the capacity to mimic the complex architecture of full human skin, it represents a valuable alternative since its composition closely resemble that of the *SC* layer and the results obtained using Strat-M® are highly reproducible due to the simple nature and standardized construction of this model (Yun et al., 2018).

These commercially available skin models have been used for several purposes, namely for the evaluation of the permeability of drug as well for irritation and toxicological studies (Alépée et al., 2015, Alépée et al., 2014, Kandarova et al., 2004, Kandarova et al., 2005, Kandarova et al., 2006). In the literature, several studies have compared LSE and HRE systems with animal and human skin models and the results pointed out their applicability as skin mimetic systems for the evaluation of skin absorption, testing of cosmetic formulations and for toxicological studies (van Ravenzwaay and Leibold, 2004, Schafer-Korting et al., 2008, Schreiber et al., 2005). Despite the fact that cell-based models can simulate better the human skin, due to the presence of human cell in their composition, these models present some disadvantages, namely due to the lack of skin appendages, their high cost of production and the extremely short shelf time (Flaten et al., 2015, Netzlaff et al., 2005).

A summary of the main advantages and disadvantages of the *in vitro* models reviewed in the last subsection is reported in Table 3.

Table 3 – Summary of the main advantages and disadvantages of silicone model membranes, PAMPA, PVPA and cell-based skin equivalents models.

Skin model	Advantages	Limitations
Silicone model membranes	Reproducibility Low cost Prolonged storage stability	Non-lipid-based Low resemblance to SC Non-biological origin Lack of skin appendages
PAMPA	Reproducibility Low cost Prolonged storage stability	Synthetic lipids/non-lipid-based Lipid organization not characterized Low resemblance to SC Non-biological origin Lack of skin appendages
PVPA	Reproducibility Lipid composition easily modified Relatively low cost Storage	Lipid organization not characterized Non-biological origin Lack of skin appendages
Cell-based models	Use of human cell cultures Similarity with cellular composition of human skin Permeability similar to human skin	High cost Lack of skin appendages

Skin disease's models

Skin is not only one of the first barriers between the body and the environment but also a common site for local administration of treatments towards skin diseases. The study of the toxicological and permeation profile of drugs in skin disease scenarios is crucial however, the availability of excised human diseased skin is limited mainly due to the increasing regulatory restrictions on the use of (sick) animals and humans.

Models representing heathy skin are a good approach for testing the action of topically applied drugs however, these models do not reflect the skin with the altered morphological and physiological characteristics caused by a disease, a fact that may influence the results and the conclusions of the studies. Accordingly, the

development of skin disease's models as a valuable alternative represents a big challenge and the modification of the existent mimetic models for healthy skin can be considered a possible strategy in order to design mimetics of the altered skin, embracing the diseases' characteristics. In the last years, many models have been created for the investigation of drugs interaction with skin affected by several disorders such as alterations of skin pigmentation, photodamage (photodermatitis), inflammatory disorders (psoriasis and atopic dermatitis), cutaneous wounds and skin cancer (melanoma) (see references (Abd et al., 2016, Yun et al., 2018, Amelian et al., 2017, Randall et al., 2018) for reviews).

Similarly with healthy skin models, many types of skin models for diseases are found in the literature namely the lipid systems such as the modified skin PVPA membranes. These models can be produced considering different degrees of leakiness in order to potentially represent different degrees of compromised skin (Engesland et al., 2013). Later, the same research group used the altered PVPA membranes presenting reduced barrier functionality to allow the investigation of the permeation of a set of drugs through compromised skin (Engesland et al., 2016). The alterations in the membrane were performed by changing the volume of liposomes in the top layer in order to reduce the thickness of the barrier or by using ethanol in the preparation liposomes to generate different degrees of leakiness (Engesland et al., 2016). More skin disease models have been reported and they can comprise both *in vitro* cell-based skin substituents and *in vivo*animal models, namely based on modified mouse or guinea pig organisms. Research has been focus on the development and application of *in vivo* models for several diseases such as psoriasis, atopic dermatitis, dermatophytosis and carcinoma, as reviewed in (Abd et al., 2016, Faway et al., 2018, Randall et al., 2018, Bocheńska et al., 2017, Sarkiri et al., 2019, Coricovac et al., 2018). *In vivo* and *in vitro*models developed for skin diseases will be assessed in the following sections according to the type of disease.

Psoriatic models

A substantial number of genetically engineered mice, reviewed in (Abd et al., 2016), was developed to be used as skin diseases models and in particular some of them have been studied as *in vivo* models of psoriasis (Bocheńska et al., 2017). An example is the development of the epidermal vascular endothelial growth factor (VEGF)-knockout mice which were considered a psoriasis model and were used to identify a specific role for epidermal VEGF in the permeability-barrier homeostasis maintenance (Elias et al., 2008). Knockout mice for c-Jun and JunB proteins exhibiting skin with hallmarks common for psoriasis have been developed, since these factors are important for the differentiation of epithelial cells (Szabowski et al., 2000). Additionally, overexpression of IL-1α cytokine in the murine epidermis leads to increased proinflammatory scenario and thus these mice can be considered an interesting model (Groves et al., 1995). Knockout mice of the IL-1 receptor antagonist exhibited the development of an inflammatory response similar to those verified in human psoriatic skin being considered an useful psoriatic model (Shepherd et al., 2004).

Cell-based *in vitro* systems have been described in order to simulate compromised skin, and in generally, they are developed in-house by researcher groups. Most models have been described to mimic skin inflammatory diseases, like the approaches developed to simulate psoriasis scenarios, as extensively reviewed in (Bocheńska et al., 2017, Yun et al., 2018). An example is the work published by Chiricozzi*et al* in which a full-thickness skin model closely resembling*in vivo* epidermal architecture was used to identify cytokine-responsive genes in psoriasis and the effect of cytokine antagonists (Chiricozzi et al., 2014). Another study described the design of a human psoriatic skin equivalent used to study cytokine-induced gene expression and the effect of different drugs in the disease context (Tjabringa et al., 2008).

Atopic dermatitis models

Regarding atopic dermatitis, one of the most frequently used *in vivo* model is the NC/Nga mouse (Matsuda et al., 1997, Tanaka et al., 2012, Vestergaard et al., 1999). These animals spontaneously develop skin lesions when housed under conventional conditions, which closely resemble those found in humans. Another *in vivo* atopic dermatitis model is the flaky tail (ma/maFlgft/ft) mouse which express mutations in the genes involved in the development of the atopic-like skin lesions (LANE, 1972, Moniaga et al., 2010). In addition, histamine H4 receptor-knockout mice were developed to be used as a model for atopic dermatitis and the

results obtained in studies using this model pointed out the importance of this receptor as a potential therapeutic target for atopic dermatitis. However, some of these models exhibited distinct atopic dermatitis profiles when compared with that characteristic of the disease in humans, as discussed in (Löwa et al., 2018). Recently, a report described the use of oxazolone-induced hairless mice for the study of a new treatments for atopic dermatitis (Moner et al., 2018).

As an alternative to *in vivo* models, some *in vitro* atopic dermatitis mimetic models were described (reviewed in (Löwa et al., 2018, Randall et al., 2018, Huet et al., 2018) such as in the work from Pendaries and co-workers (Pendaries et al., 2014) where a 3D reconstructed human epidermis model was designed and used to investigate filaggrin expression in the epidermis of atopic patients and showing that downregulation occurs and can justify some of the disease related alterations. Other study reports the design and characterization of a compromised reconstructed epidermis model mimicking atopic dermatitis scenario (Rouaud-Tinguely et al., 2015). A multicell-type 3D model to mimic atopic dermatitis which includes human foreskin fibroblasts, human keratinocytes, memory-effector (CD45RO+) T cells, collagen type I and fibronectin was also reported (Engelhart et al., 2005). To study the effect of the exposure to UV light to the formation of wrinkles and discoloration process, a full-thickness skin model that mimics photodermatitis disease has been used (Kuchler et al., 2011).

Dermatophytosis models

A skin disease model for dermatophytosis was reported by Cambier*et al.* using an experimental mouse model for the study of the fungal infections in the skin (Cambier et al., 2014). Other experimental models were designed to study dermatophytosis and to evaluate the efficacy of potential antifungal treatments, as reviewed in (Faway et al., 2018). For instance, the antifungal effect of terbinafine in a reconstructed tissue have been described (Rashid et al., 1995) and another study regarding *Candida albicans* (Green et al., 2004) and reconstructed epidermis has demonstrated the potential of this type of models for the control of dermatophytosis. In addition, an *in vitro* model of dermatophytosis using arthroconidia and reconstructed feline epidermis was developed in order to investigate the efficacy of a set of antifungal molecules (Tabart et al., 2008).

Skin cancer models

Concerning on skin cancer mimetic models, several mouse models have been used to mimic melanoma and other common skin cancers, like squamous cell carcinoma and basal cell carcinoma (extensively reviewed in (Abd et al., 2016, Coricovac et al., 2018)). The work performed by Burns and co-works described an example of a squamous cell carcinoma mouse model in which the skin of SKH1 hairless mice is exposed to UVB irradiation and used to study the potential activity of anti-carcinoma drugs (Burns et al., 2013). Cozzi *et al* (Cozzi et al., 2013), Singh *et al*(Singh et al., 2015) and Wang *et al* (Wang et al., 2013) used the same mouse model to investigate the effect of different drugs in the treatment of squamous cell carcinoma. Other mouse models were developed to assess the effect of antitumor signalling inhibitors for the pathways of basal cell carcinoma (Filocamo et al., 2016, Tang et al., 2011), while xenograft models were applied in the evaluation of the activity of potential anti-melanoma drugs (Schroder et al., 2016, Chen et al., 2012, Yu et al., 2016), and a hairless mouse model which spontaneously develops cutaneous malignant melanoma has been reported (Thang et al., 2012).

Moreover, chimeric models are reported in the literature in which living human skin is transplanted onto the skin of severe combined immunodeficient (SCID) mice allowing the study of the effect of drugs in living human skin. Kundu-Raychaudhuri and co-workers have used this approach to develop a human psoriatic model to study a potential treatment for psoriasis (Kundu-Raychaudhuri et al., 2014). Likewise, SCID mouse–human melanoma models were described for the inspection of different cancer targets and therapies as reported in (Salton et al., 2015, Yue et al., 2015).

Design of an *in vitro* skin cancer mimetic models may be complex as it includes the incorporation of various tumour entities in a 3D skin system to resemble cell-cell and cell-ECM interactions (Marconi et al., 2018). As representative examples, Li *et al* described a 3D human skin reconstructed model which includes

cultured melanocytic cells (Li et al., 2011), while melanoma cells (A375), normal human-derived epidermal keratinocytes, normal human-derived dermal fibroblasts and collagen type I were assembled to simulate a metastatic melanoma (Mohapatra et al., 2007); and later on, Commandeur and co-workers proposed a skin squamous carcinoma mimetic model with squamous carcinoma cell lines (SCC12B2 and SCC13 cell lines), and normal human-derived epidermal keratinocytes, normal human-derived dermal fibroblasts and collagen type I (Commandeur et al., 2012). Moreover, Vörsmann *et al* designed a human 3D melanoma model, which includes primary keratinocytes and fibroblasts embedded into a collagen I scaffold and different types of cancer cell lines such as SBCL2 (RGP), WM-115 (VGP) 451-LU (MM) cells, in order to mimic *in vivo* tumour environment, and showed *in vivo* -like responses (Vorsmann et al., 2013).

Commercially available skin disease models

In addition to in-house developed systems, some disease's models are already commercially available, such as MelanoDerm®, Melanoma®, Psoriasis® and "Psoriasis Like" products. These models can be applied to the screening of new drugs, as reviewed in (Amelian et al., 2017).

MelanoDerm® composition includes normal human-derived epidermal keratinocytes and normal human melanocytes and it has been used for the screening of the effect of topically applied agents to prevent UVB-induced DNA damage (Passeron et al., 2009, Li et al., 2011). Melanoma® represents a full-thickness skin cancer model consisting of human malignant melanoma cells (A375), normal human-derived epidermal keratinocytes and normal human-derived dermal fibroblasts and the use of this model has been described for the investigation of some potential active anti-melanoma drugs (Li et al., 2011, Ma et al., 2008). The composition of the commercially available Psoriasis® model includes normal human-derived epidermal keratinocytes and psoriatic dermal fibroblasts, expressing psoriasis-specific markers and releasing psoriasis-specific proinflammatory cytokines and the model allows the study of the psoriasis biology phenomena and to screening of anti-psoriasis drugs . "Psoriasis Like" consists in normal human-derived epidermal keratinocytes cultured in a special medium to induce a diseased psoriatic phenotype, namely the destabilization of the epidermis (Desmet et al., 2017).

These commercially available models can represent a valuable alternative to in-house developed systems probably leading to more reproducible results. However, their price and the low shelf storage time are some of the possible disadvantages of these approaches. Nevertheless, these alternatives may be useful in the understanding of the role of several skin diseases as well in the evaluation of new targets and potential treatments for some skin disorders.

New trends in skin models engineering

Despite the great developments done concerning *in vitro* lipid- or cell-based models, the demand for new and more realistic human skin models still remains. Thus, and following the most recent advances in 3D bioprinting technology, production of bioprinted skin has been reported for skin engineering field. Bioprinting has been used for the fabrication of several tissues and organ models, and skin is not an exception (reviewed in (Randall et al., 2018, Yun et al., 2018, Weinhart et al., 2019, Satpathy et al., 2018, Tarassoli et al., 2018)). Bioprinting is now considered a promising fabrication method to produce skin equivalents as it allows the obtention of multilayered and multicellular system.

The production of bioprinted skin using these new approaches comprises a computer-controlled deposition of skin cells and matrix polymers following spatially controlled patterns, thus controlling the architecture of the skin model with high reproducibility and therefore revealing great potential to mimic this human organ (Randall et al., 2018).

Complex human skin models with appropriate cell compositions and matrix structure could be biofabricated through different 3D printing techniques such as electrospinning, microextrusion, ink-jet printing and laser-assisted bioprinting (reviewed in (Yu et al., 2019, Randall et al., 2018, Yun et al., 2018)). The selection of the most adequate printing technique is usually determined by the type of the biomaterials chosen for the mimetic model. The variety of available printing technologies has provided multiple options to fine tune the

structure of the model according to the desired application.

In particular, with the electrospinning technique, different voltages are applied to the polymer solution in order to generate filaments which are therefore deposited into a surface. In microextrusion printing, the polymer solution passes through a needle, is deposited layer-by-layer on the platform and is possible to assemble multiple layers by controlling the needle movement. Alternatively, ink-jet printing methods allow the dropwise deposition of the bioink and the droplets can be generated considering temperature or pressure variations. Laser-assisted bioprinting approaches comprise the use of a laser beam is which is pulsed on top of the donor layer containing the desired bioink formulation and thus leading to the creation of bioink droplets which are further deposited in the acceptor surface. The fine-tune of the laser position allow the construction of a model with a of the desired pattern (Yu et al., 2019).

In the last years, many studies reported the successful use of this method to obtain skin mimetic models. As an example, a direct cell printing method was used to produce multilayered models containing fibroblasts, keratinocytes and a collagen-based hydrogel as the structural components to mimic skin layers (Lee et al., 2014). In another study, the authors considered the use of a mixture of collagen/ fibroblasts as the bioink (i.e. a substance composed by living cells and/or polymers that can be used for 3D printing of the models) and the consecutive deposition of melanocytes and keratinocytes to obtain functional skin constructs (Min et al., 2018). Koch *et al* . reported the development of a model in which keratinocytes and fibroblasts were embedded in a collagen/Matrigel® matrix and results have shown that cells were able to express connexins, pan-cadherin and laminin (Koch et al., 2012).

In addition to bioprinting technics, other complex next-generation skin models are reported in the literature, namely regarding microfluidic technology, the called "skin-on-a-chip" devices (Rademacher et al., 2018, van den Broek et al., 2017, Zhang et al., 2018, Sriram et al., 2018). Skin-on-a-chip systems comprise the growing of different cells at a microscale environment using a microfluidic culture device in which is possible a dynamic perfusion and controlled ventilation, thus presenting many advantages namely in epidermal morphogenesis and differentiation. However, due to the high costs and technical requirements associated with microfluidics devices, the use of this new approaches is still limited and thus reinforcing the need of further investigations in this field in order to optimize these methods and allow the overcoming of their disadvantages.

In summary, the production of readily accessible and reproducible constructs for use in research laboratories, with high durability and at a low price is still aimed. Some studies have already reported the first steps considering these challenges (reviewed in (Abaci et al., 2017)) however it is expected that further studies in this field of research can solve the existent drawback of the available models.

Conclusions

In face to the strict legislation regarding human and animal tests and in particular to the urgent need of development of efficient skin mimetic models for the evaluation of new skin pharmaceutical drugs and cosmetics, without using animal models, many different skin substituents have been described in the literature. The numerous examples mentioned in the present review highlighted the many different types of models from the most simple non-lipid approaches to the lipid-based models or even cell-based *in vitro* systems or at least *ex vivo* human or animals skin mimetics. In a lesser extent, some skin disease models have been reported aiming to mimic the morphological and functional characteristics of compromised skin, namely for psoriasis, atopic dermatitis or melanoma diseases.

Several improvements in the quality, complexity and mimetic properties of the skin mimetic models have been accomplished as well as the use of different technologies to produce the models and to simulate the highly complex and stratified structure of the human skin. Yet, some big challenges persist such as: a) the need of reproducibility in the results obtained using these models; b) the capacity of the models to better mimic the multitude of human skin structure and functions; c) the applicability of the use of that models to the evaluation of drugs exhibiting distinct physicochemical properties d) the improvement of storage conditions and shelf time and e) to guarantee the production of cost-effective skin membrane models.

With the last advances promoted by the use of advanced skin engineering technologies, the incorporation of various skin highly complex components such as vascularization, innervation, pigments and immune cells and other complex structures like glands or hairs in these models is started to be possible.

In the future it is expected that the models can evolve towards the "perfect" skin mimetic model, overcoming the drawbacks of the already described and allowing the efficient screening of new drugs and cosmetics, in a heathy skin or in a disease scenario. However, the selection of the most useful and adequate model(s) will probably be always a crucial point in the design of a study to test skin agents since the nature and complexity of the different described systems can be largely distinct. In the selection process of the best skin mimetic model several aspects may be considered as for example the main aim of the case study, the physicochemical properties of the drugs of interest, the type of biological effect expected for the compounds, amongst other important factors, namely those regarding the characteristics of the existent mimetic skin models such as their availability in the market, quality, complexity, stability, price and mimetic properties of each skin alternative.

In sum, perhaps the concomitant use of different skin mimetic models can allow a superior understanding of each case of study, thus revealing many different conclusions which probably cannot be obtained if a unique skin mimetic model was considered.

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