
Book Section:
Ribeiro, A.R., Costa, S., Nogueira, S. et al. (5 more authors) (2025) Advanced skin models for nanomaterials safety assessment. In: Alfaro-Moreno, E. and Murphy, F., (eds.) Nanosafety: A Comprehensive Approach to Assess Nanomaterial Exposure on the Environment and Health. Springer Cham, pp. 161-191. ISBN: 9783031938702.

https://doi.org/10.1007/978-3-031-93871-9_7

Advanced Skin Models for Nanomaterials Safety Assessment

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Abstract

The human skin acts as a biological shield against prolonged exposure to nanomaterials (NMs) and nanoparticles (NPs) coming from cosmetics, textiles, and environmental pollutants that are known to lead to adverse effects such as oxidative stress, skin irritation, and skin diseases. This chapter reviews the main pollutants that our skin are exposed to daily as well as the advanced *in vitro* skin models used for assessing nanotoxicity. It is widely known that the existing 2D and 3D skin models try to mimic the complexity of skin physiology however they still lack specific skin structures such as vascularization and hair follicles. Skin-on-a-chip (SoC) devices, employing microfluidic technologies, bring the advantage of offering dynamic environments for more realistic evaluations of NMs' safety assessment. In this chapter, we analyze critically how these models could accelerate nanotoxicity testing and support regulatory decisions. Additionally, we also review existing biological assays for skin toxicity as well as the available computational models (*e.g.,* Nano-QSR) that could help in

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E. Alfaro-Moreno, F. Murphy (eds.), *Nanosafety*,
https://doi.org/10.1007/978-3-031-93871-9_7

predicting nanotoxicity taking into consideration the physicochemical properties of NMs. Future research should focus on enhancing skin model complexity and employing computational methods to predict NM behavior, ensuring the safe development of nanomaterials for dermal applications.

Keywords

Nanomaterials · *In vitro* and computational models · Nanotoxicology · Safety assessment

1 Skin a Route of Exposure to NMs

The skin serves as the primary barrier against repeated and persistent stressors, such as nanomaterials (NMs) and nanoparticles (NPs) (Domingues et al. 2022; Gupta et al. 2022; Parrado et al. 2019) Exposure occurs through interaction with NM-enabled consumer products (such as textiles, cosmetics, tattoos, and drug delivery systems), emissions from anthropogenic industrial processes that release NMs into the environment (*e.g.,* pollutants), and occupational exposure, where construction workers, painters, hairdressers, manufacturing, and laboratory personnel are in continuous contact with NMs (Asmatulu et al. 2022; Domingues et al. 2022; Gupta et al. 2022; Omari Shekaftik et al. 2022; Wang et al. 2022).

Regarding consumer exposure, the market offers many cosmetic products containing NPs (see Table 7.1). For example, inorganic NPs (metals and their oxides) are highly stable, hydrophilic, biocompatible, and typically non-toxic. These properties make them frequently employed in sunscreen applications (*e.g.,* titanium dioxide and zinc oxide) due to their effective UV radiation filtering properties (Chauhan and Chauhan 2021). Moreover, silver NPs possess broad antimicrobial activity and are often used in cosmetic products due to their preservative effects, particularly in acne formulations. They are also incorporated into topical medical treatments for burns as an anti-infective agent (Asmatulu et al. 2022; Domingues et al. 2022; Gupta et al. 2022; Omari Shekaftik et al. 2022; Wang et al. 2022). Silicon dioxide NPs are applied in cosmetic formulations and as drug carriers, commonly recognized in rinse-off and leave-on cosmetic products for hair and face (Asmatulu et al. 2022; Domingues et al. 2022; Gupta et al. 2022; Omari Shekaftik et al. 2022; Wang et al. 2022). In recent years, the presence of gold NPs in the cosmetics market has increased significantly, as they stimulate collagen secretion and cell regeneration, providing a glow and preventing skin wrinkling (Mascarenhas-Melo et al. 2023). Organic particles such as micelles, liposomes, and dendrimers are considered non-toxic, and biodegradable, with their shapes (nanospheres or nanocapsules) ideal for transdermal uptake. Carbon-based particles such as fullerenes, carbon fibers, and graphene have been employed in skin applications to enhance the delivery of cosmetic agents (Asmatulu et al. 2022; Domingues et al. 2022; Gupta et al. 2022; Omari Shekaftik et al. 2022; Wang et al. 2022). Besides exposure to consumer products, the skin is also exposed daily to air pollutants (a multi-component mixture that includes particulate matter (metals, organic compounds, inorganic

Table 7.1 Overview of reported NP and NMs present in consumer products and occupational settings

Products occupational settings	NMs/NPs	Applications and properties	Potential effects on the skin
Textiles	Carbon nanofibers	Increase textiles' tensile strength, durability, and thermal stability.	Prolonged exposure might cause skin irritation and/or allergic reactions (Bacakova et al. 2020)
	Nanoclays	Improve textiles resistance to fire, water, and gases.	Potential toxicity (Saleem and Zaidi 2020)
	Ag	Inhibit bacteria, fungi, and other microorganisms' growth.	Prolonged exposure might cause skin irritation, allergic reactions and alter skin microbiome (Koivisto et al. 2024; Melnik et al. 2023)
	Au	Thermal conductivity, optical, and antimicrobial properties, employed in smart textiles that integrate electronic components or sensors.	Prolonged exposure might cause skin sensitivity or allergic reactions (Rosie Broadhead 2023)
	Cu	Textiles for medical or therapeutic applications due to their antimicrobial and anti-inflammatory properties.	Persistent exposure might cause skin irritation or allergic reaction and can disrupt skin microbiota (Broadhead et al. 2021)
	TiO_2	Provide UV protection and durability.	Continued exposure might cause skin irritation, leading to redness and swelling (Rashid et al. 2021)
Cosmetics	TiO_2	Sunscreens, lip balms, and makeup products such as powders and blushes, creams, and lotions to provide UV protection and transparency to the formulations.	Skin penetration in damaged or compromised skin (Lee et al. 2020a)
	ZnO	Sunscreens, makeup products such as powders, ointments, creams, and acne treatment products to provide UV protection, control shine and oiliness on the skin, having antimicrobial and anti-inflammatory properties.	Potential increase oxidative stress markers, which might have implications for skin health and can disrupt the natural microbiota of the skin (Lee et al. 2020a)
	CeO_2	Sunscreens since it provides UV protection, and antioxidant properties.	Can induce oxidative stress and affect cellular health (Ali et al. 2015)
	Al_2O_3	Sunscreens, creams, and exfoliants to improve cosmetics' texture and remove dead skin cells.	High concentrations can cause skin irritation (Dobler et al. 2019)
	MgO	Creams for oily skin, and makeup products such as powders and blushes to control shine and oiliness on the skin, improve cosmetics' texture.	Potential to disrupt the natural balance of skin microbiota (Dobler et al. 2019)
	Au	Skincare products to prevent loss of collagen and elastin and protect from free radicals.	Skin irritation or allergic reactions (Liu et al. 2022)

(continued)

Table 7.1 (continued)

Products occupational settings	NMs/NPs	Applications and properties	Potential effects on the skin
Tattoos	Quantum dots	Tattoo inks to create vibrant colors, and due to their photostability.	Skin penetration with the potential to enter the bloodstream and cause systemic effects (Ryman-Rasmussen et al. 2006)
	Carbon nanotubes	Provide stability of the tattoo ink, reducing fading over time, and create tattoos with fine lines and precision.	Skin inflammation, or allergic reactions (Battistini et al. 2020)
	TiO_2	White pigment in tattoo inks, enhancing the contrast of tattoos, and providing resistance to fading.	Skin inflammatory, or allergic reactions (Battistini et al. 2020)
	ZnO	White pigment in tattoo inks, with antibacterial properties, which can reduce the risk of infection.	Skin irritation, sensitization, and phototoxicity (Jang et al. 2012)
	Ag	Metallic or reflective effects in tattoos.	Skin irritation and allergic reactions such as redness, itching, and swelling (Islam et al. 2016)
Construction workers	Nanoclays	Mechanical improvement of mortars.	Long exposure cause skin irritation, redness, itching, dryness (Ferreira et al. 2023)
	TiO_2	Mortars to confer abrasion resistance and cement hydration, in glass to confer fouling resistance, and in paints to confer UV resistance.	Can induce oxidative stress and affect cellular health (Ferreira et al. 2023; Malte et al. 2020)
	Fe_2O_3	Mortars for electrical conductivity, mechanical improvement, and permeability reduction.	Skin irritation and allergic reactions and induce oxidative stress (Ferreira et al. 2023)
	SiO_2	Mortars to improve abrasion and freeze-thaw resistance, in roads for mechanical improvement.	Prolonged exposure might cause skin irritation, leading to redness, itching, and dryness (Ferreira et al. 2023; Malte et al. 2020)
	$Ca(OH)_2$	Wall paintings, mortars, renders, and plaster due to biocidal activity providing protection.	prolonged exposure can cause severe skin irritation and even chemical burns (Ferreira et al. 2023; Malte et al. 2020)

(continued)

Table 7.1 (continued)

Products occupational settings	NMs/NPs	Applications and properties	Potential effects on the skin
Hairdressers	TiO_2	Hair care products for their UV-blocking properties.	Skin penetration and induction of oxidative stress and inflammatory responses (Malte et al. 2020; Rosen et al. 2015)
	SiO_2	Hair products for their conditioning properties.	Prolonged exposure can lead to skin irritation (Rosen et al. 2015)
	Ag	Hair products for their antimicrobial properties.	Prolonged exposure can cause argyria and other toxic effects (Rosen et al. 2015)
Manufacturing and laboratory personnel	Carbon nanotubes	Production of conductive films and electronic displays.	Skin irritation and inflammation (Hu et al. 2015)
	Au	Diagnostic tests and drug delivery systems.	High concentrations can cause oxidative stress and inflammation (Dhasmana et al. 2017)
	Fe_2O_3	Magnetic resonance imaging, drug delivery, and hyperthermia treatment for cancer.	Skin irritation, inflammation and induce oxidative stress (Dhasmana et al. 2017)
Anthropogenic industrial processes	Ultrafine particles	Emitted from vehicles and industrial combustion processes.	Skin penetration, oxidative stress and prolonged exposure can cause skin aging (Dijkhoff et al. 2020)

carbonaceous material, sulfate, and nitrate), nitrogen oxides, polyaromatic hydrocarbons, nicotine, formaldehyde, and microorganisms) from sources such as wildfires, factory emissions, automobile exhaust, and smoke. These pollutants vary in time and space, and it is known that they induce oxidative stress, impair skin barrier function and ultimately lead to the development of skin diseases (Gu et al. 2024).

Due to its distinctive location, human skin works as a biological shield against NMs, where prolonged and repetitive exposure has been demonstrated to have severe deleterious effects on cutaneous tissue. The human skin is essential for maintaining body temperature, preventing the loss of water, inhibiting the entry of bacteria and xenobiotics, and performing metabolic processes (Larese Filon et al. 2015). Advanced interactions between skin cells and the microbiota establish protective skin mechanisms, where a mechanosensory system continuously detects and reacts to different external stimuli, including NMs (Pelikh et al. 2021; Shapira et al. 2022). The skin is divided into hypodermis, dermis, and epidermis where the outermost layer of the epidermis, called stratum corneum (SC), is responsible for the skin barrier function (see Fig. 7.1) (Gupta et al. 2022; Larese Filon et al. 2015; Sanches et al. 2020). The deepest subcutaneous layer, known as the hypodermis, is made up of loose, fatty connective tissues that support the dermis. The dermis layer, which supports the epidermis and skin appendages, is the skin's vascularized elastic

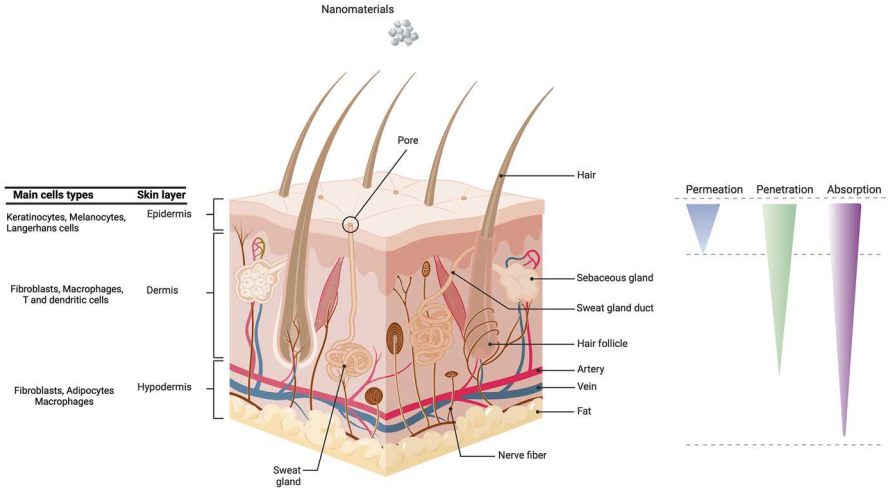

Fig. 7.1 Schematic diagram of the three fundamental skin structural layers and the main cell types found within each layer. (Constructed in Biorender)

connective tissue. Rich in fibroblasts, collagen fibres, elastin, and proteoglycans that form an extracellular matrix (ECM) where immune cells (mast cells, lymphocytes, and macrophages) adhere to (Gupta et al. 2022; Larese Filon et al. 2015; Sanches et al. 2020). The dermis also includes sympathetic fibres, sweat glands, and blood vessels that fund the dermis sensory system. Three cellular types make up the epidermis: melanocytes, Langerhans cells (LC, a type of specialized immune cell), and undifferentiated keratinocytes. As they go towards the outer layers, keratinocytes go through a differentiation process that involves morphological and biochemical alterations that drive cells from an undifferentiated, proliferative state toward metabolic inactivity. The epidermis is innervated by subepidermal nerve bundles and intraepidermal nerve fibres. The distinct qualities of the human skin barrier are established by the skin lipid matrix, which is made up of ceramides, cholesterol, and fatty acids arranged in an orthorhombic lattice (Gupta et al. 2022; Larese Filon et al. 2015; Sanches et al. 2020). Resuming the cutaneous microenvironment is constituted by microbial, immunological, chemical, and physical barriers that preserve skin homeostasis (Parrado et al. 2019).

When considering transdermal absorption of NPs, it can be defined as their ability to reach the circulatory system through penetration across various skin layers, typically occurring via passive diffusion following Fick's Law. Consequently, NPs can become systemically available and accumulate in other organs (*e.g.*, liver and spleen) (Alkilani et al. 2015). Additionally, skin permeation refers to the diffusion of NMs into a specific skin layer, whereas skin penetration involves diffusion into deeper layers, as illustrated in Fig. 7.1 (Alkilani et al. 2015). The *in vitro* and *in vivo data* on the potential dermal absorption and/or penetration of NPs exhibit controversial results. Although several studies report contrary findings, NPs penetration in both healthy and compromised skin (*e.g.,* scarring, sunburn, and depilated skin) has

been demonstrated within the scientific community (Sanches et al. 2020). The main pathways of NPs penetration are intracellular, intercellular, and transappendageal. The intercellular route is the most prevalent pathway, wherein NPs cross the stratum corneum by diffusion among the cells. For this pathway, the size, as well as the mechanical properties of NPs, need to be taken into consideration, as they need to have the right flexibility. Rigid particles, such as metal NPs, have been found to scarcely penetrate the SC via the intercellular route due to their lack of flexibility which hinders their diffusion between the cells. On the other side, the intracellular pathway is challenging as the NPs must overcome both the lipophilic (cell membrane and the lipid matrix) and lipophobic structures (inside the cells) within the skin cells (Asmatulu et al. 2022; Domingues et al. 2022; Gupta et al. 2022; Larese Filon et al. 2016; Niska et al. 2018; Salvioni et al. 2021; Tordesillas et al. 2018). NPs pass through both the lipid bilayer and the cytoplasm of keratinocytes diffusing through the corneocytes of the SC (Asmatulu et al. 2022; Domingues et al. 2022; Gupta et al. 2022; Larese Filon et al. 2016; Niska et al. 2018; Salvioni et al. 2021; Tordesillas et al. 2018). Nanocarriers with a definite degree of amphiphilicity may be worthy candidates to avoid this difficulty. In contrast, skin appendages such as hair follicles (HF), sebaceous glands, and sweat glands have been identified as potential alternative entry routes. First, NPs tend to accumulate in HFs, where they may persist for several days or weeks (Sun et al. 2018; Yuan et al. 2019). Second, due to the presence of the SC in the lower regions of HFs, there is an increased likelihood that NPs will penetrate the surrounding tissues or be taken up by specialized immune cells, such as macrophages, Langerhans cells (LCs), and dermal dendritic cells. Once activated, these cells can either function locally by clearing NPs and secreting specialized immune mediators or migrate to lymph nodes to initiate an adaptive immune response (Sun et al. 2018; Yuan et al. 2019). The HF interface with lymph and blood streams through capillary vessels is the quickest and most efficient mechanism for NPs to enter the systemic circulation. A high density of Langerhans cells around hair follicles, were capable of internalize NPs of various sizes, whereas the transport across the epidermis was restricted to 40 nm particles. For rigid NPs, the most projected path for penetrating the skin is via the follicular route. It is important to stress that the transappendageal pathway is limited, as HF and glandular ducts make up only 0.1% and 0.01% of the total surface area of the skin, respectively (Asmatulu et al. 2022; Domingues et al. 2022; Gupta et al. 2022; Larese Filon et al. 2016; Niska et al. 2018; Salvioni et al. 2021; Tordesillas et al. 2018).

NPs penetration appears to be limited to the SC due to its robust barrier formed by dermal and robust tight junctions. However, increasing evidence suggests that NPs can penetrate healthy skin, although the precise mechanisms remain unclear. The ability of NPs to penetrate the skin is influenced by the nature of formulation (containing solvents or detergents), NP's physicochemical properties such as size, shape, rigidity, and surface charge as well as skin characteristics, including follicular density, thickness, metabolism, and structural variations across different anatomical regions (Asmatulu et al. 2022; Domingues et al. 2022; Gupta et al. 2022; Larese Filon et al. 2016; Niska et al. 2018; Salvioni et al. 2021; Tordesillas et al.

2018). Additionally, aged or damaged skin due to factors such as sunburn, depilation, scaling, scarring, skin hydration, or any skin disease exhibits increased susceptibility to NPs penetration, allowing larger particles (>45 nm) to permeate more easily (Gupta et al. 2022; Palmer et al. 2019).

2 The Evolution of *In Vitro* Skin Models for Nanotoxicological Studies

The safety assessment of NPs for skin application starts with hazard identification, where the toxicological profile is undertaken via several tests (*in vivo, in vitro*), and clinical and epidemiological studies. With all this information some parameters such as no observed adverse effect level (NOAEL) and no observed effect level (NOEL) can be measured to study the exposure—toxic response, exposure assessment, and ultimately the risk characterization (Coimbra et al. 2022). Different types of safety studies can be employed to ensure the safety of consumers. The effect of NPs on the skin and their underlying mechanisms can be explored by employing *in vitro* 2D and 3D models that mimic skin physiology using genuine or synthetic skins and *in vivo* models (mice, pigs, rabbits, guinea pigs, and human explants) (Melnik et al. 2023).

Nonetheless, it is widely reported that non-clinical *in vivo* investigations of cosmetics frequently fail to translate to human clinical trials due to species-specific physiological characteristics (*e.g.*, skin thickness, cell populations, HF density, and immunology). Human skin explants are far more physiologically relevant since they contain the entire skin architecture as well as most skin cell types. However, they can be greatly influenced by individual variables (age and living patterns) limiting reproducibility, but they carry ethical concerns and availability issues due to the dependence on discarded tissue from the plastic surgery of healthy individuals (Melnik et al. 2023).

Driven by ethical concerns and societal ambition to reduce animal experimentation (prohibited by the European Cosmetic legislation) reliable and animal-free systems that try to emulate human skin have been employed. The passage of the FDA Modernization Act 2.0, as well as growing confidence in more complicated human-based *in vitro* models, is paving the way for more human-relevant research into nanotoxicity processes (see Fig. 7.2).

2.1 Two-Dimensional Models

Two-dimensional models of the dermis and epidermis have expanded the knowledge of how skin cells behave and respond to a variety of stimuli, including NPs. The most usual 2D skin models share a combination of key features such as human- or animal-derived fibroblasts, epidermal keratinocytes, and a basal supportive substrate. *In vitro,* studies demonstrate the importance of ECM proteins such as collagen fibres in maintaining dermal fibroblast integrity (Dijkhoff et al. 2020; Wistner et al.

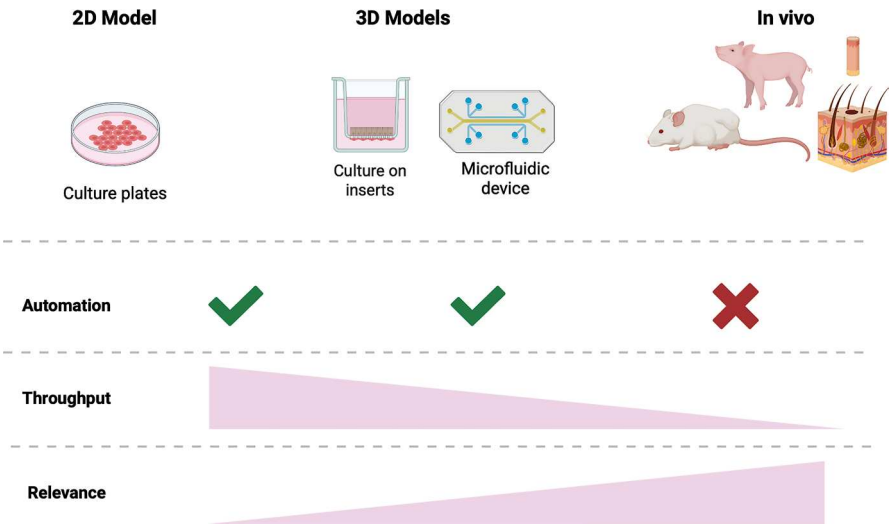

Fig. 7.2 Available skin models their possible automation and throughput relevance. (Constructed in Biorender)

2023). When skin cells are isolated from their dynamic environment and grown in stiff and static Petri dishes, they demonstrate low predictive power over clinical outcomes and as such they are limited largely to cytotoxicity studies but are useless at elucidating efficacy in drug testing (Dijkhoff et al. 2020; Wistner et al. 2023). Human adult low calcium high temperature (HaCaT) cells and recently human telomerase reverse transcriptase (hTERT)-immortalized human keratinocytes cell lines together with murine fibroblasts (3T3) are frequently employed for 2D *in vitro* studies (Alépée et al. 2014; Boukamp et al. 1988). Nevertheless, primary human cells isolated directly from the neonatal foreskin or adult skin of healthy donors are the more biologically relevant. Interestingly it was already described that keratinocytes need to be co-cultured with supporting fibroblasts or fibroblast-conditioned media to produce durable epithelium, suggesting the importance of fibroblasts-keratinocytes communication for epidermal formation, growth, and differentiation. Normal human epidermal keratinocytes (NHEK) or normal human dermal fibroblasts (NHDF) monocultures are frequently isolated and cultured on specific substrates in an optimized cell culture medium and as well as keratinocytes can receive NPs treatment directly in their culture medium. 2D models were employed in the 1980s to predict cytotoxicity (OECD 2010) and phototoxicity (in 3T3 cells; (OECD 2015)). In 2004, OECD guideline 432 was the first *in vitro* test that largely replaced animal hazard analysis. Although the usage of 2D models allows for reproducible, throughput, and low-cost experiments for the basic study of cell growth and survival of epithelial systems, they still fail to mimic skin physiology, lacking a physiological skin barrier with a competent SC layer (Abd et al. 2016; Augustine 2018; Avci et al. 2013; Cui et al. 2010; De Wever et al. 2015; Elaine 1999; Filaire et al. 2022; Guichard et al. 2022; Gupta et al. 2013; Krieg and Aumailley 2011; Kwak et al.

2020; Lee et al. 2020b; Motter Catarino et al. 2022; Pereira et al. 2013; Ramadan and Ting 2016; Venus et al. 2010; Yurchenco' and Schittny 1990; Zeb et al. 2019). For example, it has already reported that the oxidative stress and pro-inflammatory response of silver NPs were higher in a 2D keratinocytes model compared to a 3D epidermal model due to the impaired SC (Chen et al. 2019). Resuming, 2D skin models do not recapitulate the cutaneous environment, they overestimate toxicity, have limited predictive capacity, and have differences in physiology and genetic background (Domingues et al. 2022; Rogal et al. 2022; Schneider et al. 2021; Wu et al. 2020). Thus, these 2D models inspired the development of skin equivalents that employ 3D scaffolds to mimic skin structure and biological complexity (Sun et al. 2006).

2.2 RHE and Full-Thickness Models

The advent of reconstructed *in vitro* skin models marked a significant advancement in dermatological and toxicological research. Moving beyond traditional two-dimensional cell cultures, these models provide a more physiologically relevant platform to study skin biology, and disease pathogenesis, and assess the safety and efficacy of cosmetics, pharmaceuticals, and NPs/NMs (Alépée et al. 2019a, b; Hofmann et al. 2023; Moon et al. 2021; Singh et al. 2024). Reconstructed human skin models, developed as an animal-free alternative to previous models, are obtained from human cells like epidermal keratinocytes and fibroblasts, but cultured in a multilayered format (epidermis and dermis), generated by combining biomolecules such as collagen and human-derived cells. One important aspect regarding the creation of fully differentiated *in vitro* skin models is that they require culture at the air-liquid interface (ALI) (Chen and Schoen 2019). To create an ALI culture, epithelial cells are seeded in compartmentalized culture systems on the top of porous filter supports that keep them physically isolated from the underlying fluid. Typically, porous membranes are employed to divide the layers and replicate structures found in native human skin while facilitating intercellular crosstalk. After the initial attachment and proliferation phase and the creation of a confluent monolayer, the culture media on the apical side is removed. The cells 'interface' with the surrounding air, differentiate, and create an apical microenvironment through transudation and apical secretion. The basal surface of the cells has access to the culture media, including nutrients and other additives, via diffusion through the porous membrane (Chen and Schoen 2019).

The Reconstructed Human Epidermal (RHE) model consists of organized basal, spinous, and granular layers, with a multilayered stratum corneum containing intercellular lamellar lipid layers arranged in patterns, representing the main lipids found *in vivo* (Alépée et al. 2019a, b). RHE is typically constituted of keratinocytes differentiated to form a multilayered epidermis, mimicking the skin's barrier function. This permits researchers to evaluate the potential of substances to induce irritation, corrosion, sensitization, and absorption (see Table 7.2). Considering NPs, RHE models are particularly useful in evaluating their safety, providing insights into NPs

Table 7.2 *In vitro* models used for cosmetic screening employing OECD guidelines

In vitro model	Biological output	OECD	Characteristics
3T3 NRU	Phototoxicity	432	BALB/c 3T3 mouse fibroblasts to measure the concentration-dependent reduction in neutral red uptake by the cells after exposure to a test material (presence or absence of UVA).
SkinEthic™	Skin irritation	439	The biological properties of the RhE model prevent the passage of a material around the stratum corneum to the viable tissue.
KeratinoSens™	Skin sensitization	442 D	Employ immortalised cell line derived from human keratinocytes and measure luciferase gene induction as an indicator of the activity of Nrf2 transcription.
epiCS®	Skin irritation	431	The model consists of organized basal, spinous and granular layers, and a multi-layered stratum corneum containing intercellular lamellar lipid layers.
Human or animals skin	Skin absorption	428	The test system includes the donor chamber, the skin surface rinsing, the skin preparation and the receptor chamber.

penetration, uptake, and potential toxicity to epidermal layers (Moon et al. 2021). For example, EpiDerm™ skin was incubated with iron, aluminium oxide, titanium dioxide, and silver NPs and tested for skin corrosion and irritation based on the OECD TG431 and TG439. Results demonstrated that NPs were non-corrosive and non-irritant and that the *in vitro* model is suitable for NPs safety assessment. EpiSkin™ was also employed to evaluate the skin irritation caused by metallic NPs such as aluminium oxide NPs, TiO_2, and Ag NPs after short and long-term incubations and the epidermal penetration of gold NPs (Filaire et al. 2022; Hao et al. 2017; Kim et al. 2016; Strüver et al. 2017). However, the skin is a complex organ, and

RHE models, while valuable, lack the full complement of cell types and structures present *in vivo*. To address this, full-thickness skin models have been developed, where collagen matrices are widely employed and known to provide fibroblasts with an adequate environment to support ECM protein synthesis and paracrine factor secretion, thus promoting keratinocyte growth, maturation, and formation of stratified epithelium. These advanced models besides incorporating a dermal compartment, often populated with fibroblasts, may include additional cell types such as melanocytes and immune cells (Abdayem et al. 2016; Hofmann et al. 2023). The inclusion of these components allows for a more comprehensive assessment of NPs interactions with the skin, including potential inflammatory responses and immunotoxicity.

Commercially available RhE models already validated are the EpiSkin™ (L'Oreal, France), EpiDerm™ (MatTek Corporation, Massachusetts, USA), SkinEthic™ (SkinEthics, France), and epiCS® (CellSystems, Germany) (Chen et al. 2024). Indeed, some can recapitulate the epidermis, and others already replicate both dermal and epidermal compartments. When considering air pollutants, Phenion^{FT} skin equivalents were employed to evaluate the synergistic effect of ozone and particulate matter (PM). PM was observed to decrease the epidermal thickness and promote a matrix-building phenotype, while ozone was found to alter lipid homeostasis and induce inflammation (Reynolds et al. 2023). Skin barrier dysfunction, dose-dependent inflammatory reaction, and modifications in differentiation protein markers and water transport were observed upon exposure of PM to an in-house RHE (Hieda et al. 2020). All these events could eventually aggravate several skin diseases. Resuming, the use of 3D skin models, as an alternative to animal testing is validated to test chemicals and recommended when testing the cutaneous effects of NPs. However, we must keep in mind this model possesses weaker barrier properties compared to native skin, they lack cellular and biomolecular variety as well as vasculature or adnexal structures. This is still a main issue since their absence impacts skin functionality; however, efforts are underway to develop more skin-relevant models (Filaire et al. 2022). Also, adequate controls are necessary to avoid the interference of NPs on the biological assays.

2.3 Biofabrication of 3D Equivalent Models

Fabricating skin in a lab setting that recapitulates all the structural and functional aspects of a native dermis is a challenging task. Several biofabrication techniques have been applied to the development of the skin (see Fig. 7.3), highlighted next in this section with their respective advantages and disadvantages.

2.3.1 3D Bioprinting

This technique relies on the layer-by-layer deposition of bioinks to create complex 3D structures. There are different bioprinting techniques, each with its own characteristics, resolution, and print speed (Table 7.3). Inkjet Bioprinting deposits bioink onto a substrate using thermal or piezoelectric actuators at high print speed and

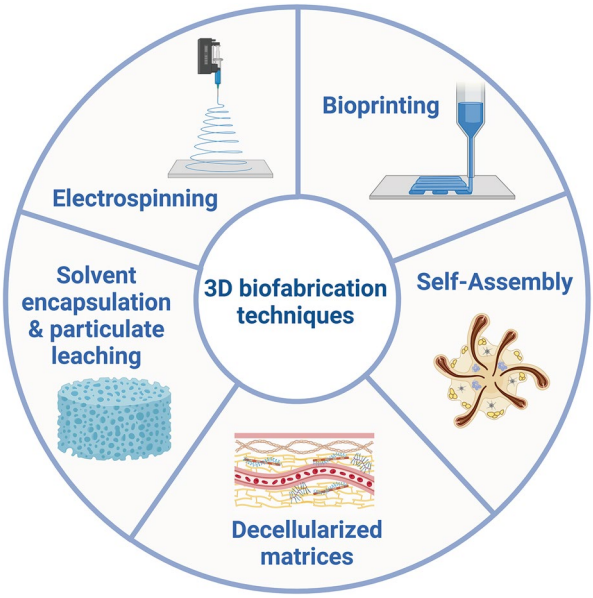

Fig. 7.3 Biofabrication techniques employed for the development of 3D *ex vivo* models of skin. (Constructed in Biorender)

Table 7.3 Types of 3D bioprinting, along with their advantages and disadvantages

Technique	Advantages	Disadvantages
Inkjet bioprinting	High resolution, fast print speed, cost-effective	Nozzle clogging, limited viscosity range, low cell viability
Extrusion bioprinting	Bioink viscosity range, scalable	Lower resolution, slow print speed, low cell viability
Laser-assisted bioprinting	High precision, high cell viability	Technically complex, expensive, slow print speed

resolution. It is therefore suitable for creating detailed patterns and depositing multiple cell types. However, it is prone to nozzle clogging, can only print with low-viscosity bioinks, and the thermal stresses involved cause low cell viability (Murphy and Atala 2014; Nakamura 2005). Extrusion bioprinting uses a continuous flow of bioink extruded through a nozzle but at a lower resolution and print speed than ink-jet bioprinting. The shear stresses experienced by cells during extrusion also decrease the viability significantly. However, it is a more versatile technique, as it can use bioinks of a range of viscosities and print larger and more complex structures (Murphy and Atala 2014). Laser-assisted bioprinting uses a laser to transfer a bioink from a donor slide to a substrate, offering excellent resolution and precision with high cell viability. However, the print speed is slow, and it is more expensive and technically demanding than the other techniques (Murphy and Atala 2014).

Overall, 3D bioprinting enables precise control over the placement of cells, biomaterials, and growth factors. Therefore, it can be used to create complex structures that recreate the multiple layers of the skin and incorporate other key structures like

hair follicles, blood vessels, sweat glands, etc. It also enables customization for patient-specific models. However, there are still limitations related to maintaining the original mechanical properties of biopolymers (like collagen) after they have been modified into bioinks, as well as the predominant animal origin of bioinks and its implications in regulatory frameworks and ethical considerations.

2.3.2 Electrospinning

Electrospinning is a technique by which nanofibers are created and deposited as scaffolds that mimic the ECM. The nanofibers are generated from both natural and synthetic polymers in a solution that is drawn from a syringe because of a high-voltage electric field, creating fine fibers. Several studies have demonstrated that electrospinning is suitable for creating dermis-like equivalents made of a range of polymers (e.g., collagen, polycaprolactone, or chitosan, amongst many others) that support the attachment and proliferation of cells (Law et al. 2017; Lizarazo-Fonseca et al. 2023; Tamilarasi et al. 2023). The main advantages of electrospinning are the high surface area for cell attachment and high porosity for nutrient and waste exchange of the resulting scaffolds. However, scaffold uniformity and, most importantly, the balancing of the mechanical properties (strength and flexibility) of the scaffolds are the most important challenges (Venugopal and Ramakrishna 2005).

2.3.3 Hydrogel-Based Encapsulation

This technique employs hydrogels, which are three-dimensional hydrophilic polymer networks that can retain large amounts of water, just like the natural ECM in the dermis. Moreover, and very much like the natural ECM, hydrogels present unique viscoelastic properties. They are also highly biocompatible and support cell attachment and growth. The large pores and high water content facilitate nutrient, oxygen, and waste exchange through diffusion. For these reasons, hydrogels are ideal biomaterials to mimic the dermis enabling full-thickness model development (Hoffman 2012; Peppas and Khare 1993).

The polymers forming the hydrogel network can be natural (like collagen, gelatin, or alginate), synthetic (like polyethylene glycol (PEG) or polyvinyl alcohol (PVA)), or a hybrid of both. Cells are suspended in the precursor solution of polymers, which are then mixed with the crosslinkers to form the 3D network. The mechanical properties (like stiffness and elasticity) of hydrogels can be easily controlled by tuning the physicochemical properties of the polymers and the ratios between polymers and crosslinkers. This enables the development of skin models of a range of mechanical properties that can mimic different diseases or processes associated with altered ECM composition of the skin. For example, scarring has been associated with higher stiffness, owing to the excessive deposition of ECM, while ageing is linked to a decrease in elasticity. Hydrogels' tunability enables the development of these types of models using the same base polymers for the scaffolds (Drury and Mooney 2003; Place et al. 2009). On the other hand, there is very little control over the cell distribution in the hydrogel, which is often not uniformly distributed within the scaffold. Moreover, it is challenging to develop complex or hierarchical structures using this technique.

2.3.4 Decellularization

Decellularization, as the name indicates, is a technique by which all cellular components from a donor tissue are removed, ideally preserving the native ECM structure, composition, and mechanical properties. By doing so, decellularization prevents immunogenicity while maintaining the scaffolding for cells to grow into new tissue. There are different methods of decellularization, divided into chemical, physical, and biological (Crapo et al. 2011; Reing et al. 2010). Chemical (detergents), physical (freeze-thaw cycles) and biological (enzymatic) methods have been widely employed to remove all cell types from the tissue, where the next step is recellularizing it, meaning repopulating the decellularized ECM with human cells to restore tissue functionality. Cells can be seeded directly on the scaffold's surface and allowed to migrate and proliferate throughout, or they can be perfused through the scaffold using bioreactors. The latter approach results in a more uniform cell distribution. The main challenges of this biofabrication technique are ensuring complete decellularization, preservation of the ECM structure and properties, and efficient recellularization of the donor tissue scaffold.

2.3.5 Self-Assembly

Self-assembly is a biofabrication technique that leverages the inherent ability of cells to organize into structured tissues. The rationale behind this is to allow cells to follow the natural processes of tissue formation and development, driven by cellular interactions, ECM production, and biochemical and biophysical cues. In this process, fibroblasts produce collagen and other ECM components to form the dermis, while keratinocytes form the epidermal layer. Self-assembly of skin models can include more cell types, like melanocytes to produce pigmentation, and endothelial and smooth muscle cells to form blood vessels (Jakab et al. 2010).

The process of self-assembly starts with a monolayer culture of the cells, followed by 3D cultures in spheroids or organoids, which promotes the self-assembly of the cells into tissue-like structures. The most obvious advantage of self-assembly biofabrication is the reduced need for synthetic scaffolds *that might cause* or degrade unpredictably. Overall, this approach reduces the hurdles for clinical applications. The resulting architecture of the *ex vivo* model is also much more physiologically relevant, compared to scaffold-based methods, and incorporates all the naturally occurring and necessary cell-cell and cell-ECM interactions. However, scaling up this biofabrication technique is very challenging, due to nutrient and oxygen diffusion limitations. Moreover, the control of tissue self-assembly of multiple cell types to produce complex tissue structures like blood vessels is also challenging, as is to incorporate these different cell types in the correct spatial organization to mimic the complex native skin.

There are many biofabrication techniques to build and mimic the complex skin architecture, each with their advantages and disadvantages, and therefore suitable for specific uses and purposes. This means that the "perfect" model does not exist, but rather a collection of models that enable the study of different aspects of the skin. For example, if only the mechanical and physicochemical environment of fibroblasts is being studied, a simple hydrogel encapsulation will suffice, providing

the ECM component and an easy platform to alter the properties and assess the effects. However, if the study aims to understand the effects of blood supply to the skin, sebum production, or hair growth on other physiological events, 3D bioprinting or self-assembly techniques that can recapitulate the complex architecture of these skin appendages and structures are key to attaining a relevant model.

2.4 Skin-on-a-Chip Models

One limitation of all the techniques highlighted in this section is that they cannot simulate the dynamic physiological environment of the skin, in particular the fluid flow and mechanical forces experienced by the tissue. Alternatively, microfluidic devices, such as skin-on-a-chip models, provide this missing aspect.

The latest advancements in microfluidics have enabled the development of organ-on-chip (OoC) devices that are established by perfused microfluidic chambers populated by cells that mimic tissue- and/or organ physiology. In OoC devices, the cellular microenvironment can be controlled with high spatiotemporal precision, allowing extracellular cues to guide cells into physiologically accurate configurations. The goal is that these OoC model cells remain viable for extended periods replicating one or a few specific functional properties of organs, such as the barrier function in the case of the skin. They can also simulate the dynamic interactions between cells, the cell-ECM, and the mechanical stresses that cells encounter within tissues (Costa et al. 2023b).

Approaches from tissue engineering are employed in skin-on-chip (SoCs), which involve cell culture on scaffolds, application of physical signals (fluid-dynamic, mechanical, electrical), and microfabrication techniques of culture spaces and channels (Costa et al. 2023b). These features enable SoCs to provide improved consistency of skin barrier function and structure while incorporating only a few cell types. In SoC systems, cells typically grow on the surfaces of microcavities or porous membranes, with continuous perfusion of medium culture supplied through the microchannels. In terms of chip design, two general trends emerge: (1) closed devices, with sealed channels and pump-driven flow; and (2) open devices that resemble well plates and are perfused by gravity-driven flow or use rocking platforms. The medium flows between microfluidic chambers via gravity, a 3D tilting mechanism, or active pumps to mimic blood flow and sustain cell viability by averting the accumulation of metabolic wastes and offering necessary fluid shear stress (Zoio and Oliva 2022). Medium flow, growth factors, and cytokines through these channels accentuate cell differentiation and augment skin tissue longevity, overcoming the limitations of pump-driven systems. Curiously, the pumpless microfluidic technology overcomes challenges, such as air bubble formation, since it provides high controllability and enables complex flow patterns, however, it can complicate device operation and cell seeding. Effective perfusion between the skin tissue and the microfluidic channel is essential for waste removal but also for tissue sustenance that is accomplished through a porous membrane (polydimethylsiloxane, polycarbonate, and polyethylene terephthalate) (Costa et al. 2023b; Zoio and

Oliva 2022). Conversely, open device chips facilitate easy tissue and media retrieval but offer less precise environmental control. To emulate skin multiple layers, the chip design should enable an air-liquid interface so that the stratum corneum layer of the epidermis is exposed to air, while the dermal layer is exposed to culture media. Simultaneously, the field of microfluidics advanced rapidly with the invention of soft lithography and the ability to prototype devices using polydimethylsiloxane (PDMS), a soft silicone-based material (Cho et al. 2024). Regarding chip fabrication, soft lithography with PDMS has been the predominant method. However, mechanical or laser-based structuring of thermoplastics polystyrene (PS), polycarbonate (PC) and polymethyl methacrylate (PMMA) are also used to overcome PDMS limitations, such as absorption of hydrophobic molecules (e.g., culture media components, NPs or tested drugs), limited scalability for industrial production, and challenges with sensor integration. PDMS advantages are the low manufacturing cost, air permeability, ease of handling, efficient sealing process, and ability to form complex micropatterns (Cho et al. 2024). All the referred materials are optically transparent and have good biocompatibility suitable for observing and performing several cell-based studies.

In recent years, numerous SoC models have been presented. These models include (i) devices where skin biopsies or reconstructed skin models are transferred to the chip, it incorporates either patient-derived skin, harvested through biopsies, or post-mortem skin samples (Costa et al. 2023b). One of the first examples is the chip designed by Abaci et al. for placing a human skin equivalent (HSE) to test its viability and maintenance. The HSE was cultured on a porous membrane to enable nutrient diffusion from the channel. The group investigated the transdermal transport of substances and the potential of this device for drug testing applications (Fig. 7.4a) (Abaci et al. 2015). The transferred SoC model more accurately represents the cellular population and interactions of the skin at a microscopic level. This method is also simpler than cultivating skin cells and creating an optimized environment within the device for long-term tissue maintenance and experimental purposes. However, obtaining sufficient skin tissue from individuals can be logistically challenging due to the extensive tissue volume requirements for research. (ii) The second strategy involves the cultivation of human skin cells on the chip the so-called in situ SoC, whereas 2D cell monolayers are cultured on porous membranes. As an example, Wufuer et al. developed an SoC model containing three layers—epidermal, dermal, and vascular—to study inflammation and edema (Wufuer et al. 2016). The layers were represented by keratinocytes, fibroblasts, and human umbilical vein endothelial cells, respectively, and were separated by transparent porous membranes to enable interlayer communication and mimic skin biology. They generated a model of skin inflammation by perfusing the chip with tumor necrosis factor (TNF-α) and measured proinflammatory cytokine levels and tight junction integrity. The efficacy of the drug dexamethasone was evaluated using this inflammation model, demonstrating that the drug could mitigate TNF-α-induced endothelial barrier dysfunction (see Fig. 7.4b). It is important to refer to that in this model the epidermis with its different layers is not achieved since the model doesn't allow culture of cells in ALI. The last approach (iii) works with platforms with perfusable

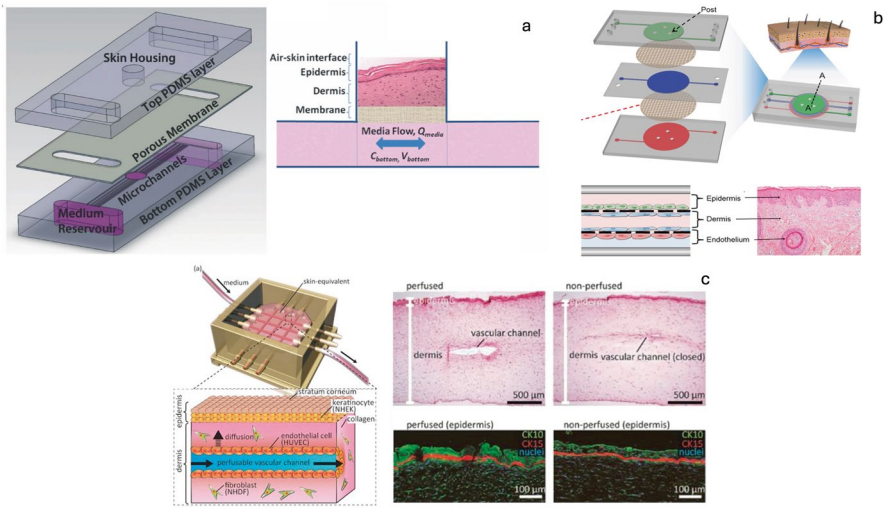

Fig. 7.4 Skin-on-chip examples: (**a**) Pumpless chip with transferred skin designed for testing HSEs viability and maintenance; (**b**) 3D schematic of the skin-on-a-chip system, which comprises three PDMS layers and two PET porous membranes, with a representative histological section of the skin; (**c**) the schematic diagram of the skin chip integrated with the perfusion vascular channel which can be used to test the toxicity of cosmetics. (Copyright permission is conveyed through Copyright Clearance Center, Inc.)

lumens (mainly for vasculogenesis) or patterned microchannels, onto which skin tissue is assembled directly using membranes or custom scaffolds. As an example, Mori et al. fabricated a culture device using 3D templating techniques (Mori et al. 2017). They developed a device with anchoring structures and nylon wires strung across connectors. A collagen structure was fixed into the device, and perfusable vascular channels were created by removing the nylon wires. This approach allowed the recreation of dermal/epidermal within a vascular channel. However, this technique lacks a complete microvascular network in the dermis. Additionally, the contraction of the collagen used for the dermal compartment affected the permeation assay, limiting it to the central portion of the (Fig. 7.4c) (Mori et al. 2017).

Resuming, most available SoC models focus on recapitulating the dermis and epidermis, primarily using cell lines. Some models also incorporate additional relevant cell types, such as vascular cells (human umbilical vein endothelial cells, human primary microvascular endothelial cells) and immune cells (human leukemic monocyte lymphoma cell line). The inclusion of vascularization in the models allows for to study NPs and drug absorption, gaining insight into the transport of intravenously injected drugs/NPs, and the opportunity to construct thick skin models with enhanced deposition of cell-derived ECM components (Rimal et al. 2024). The most reported benefit of SoC is the increased expression of filaggrin and involucrin (role in forming the epidermal skin barrier) and enhanced skin barrier function. A consistent finding is the higher mean transepithelial electrical resistance in

SoC models compared to static controls, confirming a more robust barrier with a lower permeability (Zoio and Oliva 2022).

Concerning toxicological studies, the available SoC models have yet to be investigated for NMs. Given the rising market for NM-containing products and NP-associated pollutants, monitoring toxicity has become a top issue. As a result, advanced SoC models are urgently needed to accelerate NM/NPs safety evaluation and give human-relevant data. The benefit of SoC in nanotoxicology is that tailored microfluidic channels on top of the epidermal layer could provide well-controlled and homogeneous particle exposure. The use of laminar flow profiles can be employed to mitigate the agglomeration or aggregation of NMs (Costa et al. 2023b). Besides that, SoC systems could serve as a platform for independently monitoring the characteristics of the most frequent types of NM-induced skin harm, including skin corrosion, irritation, sensitization, genotoxicity, and phototoxicity (Costa et al. 2023b).

While individual SoC models hold significant potential, coupling multiple tissue compartments in a single microfluidic circuit, known as multi-organ chips (MoCs), brings another layer of complexity. These systems enable systemic safety evaluations and the capture of metabolite toxicity (Sanches et al. 2020; Salvioni et al. 2021; Alépée et al. 2014; Boukamp et al. 1988). MoCs to capture interorgan communication between the gut and the skin as well as the liver and skin have been already developed (Chong et al. 2018). However, MoC use in understanding critical biological mechanisms related to exposure, uptake, translocation, and the effects of NMs on the skin and secondary organs remains absent.

3 Safety Testing of NM Toxicity in *In Vitro* Skin Models

As has been stated from the beginning of this chapter, the skin is a critical route of exposure for NMs and NPs and therefore a key target organ for potential adverse effects. The development and use of *in vitro* skin models have become a central and relevant aspect of the safety assessment of NMs and NPs. With these models, we can obtain valuable information on dermal toxicity while reducing the need for animal testing. When conducting NM toxicity testing in these *in vitro* skin models, several key endpoints are commonly evaluated:

- ***Cytotoxicity:***
  Cell viability assays such as the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) or LDH (lactate dehydrogenase) release assay are used to assess the cytotoxic effects of NMs on skin cells. This provides information on the concentrations at which NMs become toxic. The MTT (and similar assays) provide information related to the metabolic activity of the cells, less activity, less viability), while the LDH gives information regarding the liberation of an enzyme that should not be found outside of cells, and only being liberated once a cell is damaged and dead (Kroll et al. 2011).

- ***Oxidative stress:***
  NMs can induce oxidative stress in skin cells, leading to inflammation and other toxic effects. Assays to measure reactive oxygen species, glutathione levels, and antioxidant enzyme activity can provide insights into this mechanism of NM toxicity. Recently, it has been shown that measuring the presence of reactive oxygen species in keratinocytes, may alter the redox status of melanocytes when explored in co-culture conditions. Also, *in vitro* models have been useful in evaluating the potential protective effect of some compounds, like reveratrol (Barygina et al. 2019; Shukla et al. 2011; Soeur et al. 2015).
- ***Genotoxicity:***
  A main concern related to the use of NM in cosmetics and topical medication is the potential to cause DNA damage. *In vitro,* skin models can be used to assess NM genotoxicity using assays like the comet assay or micronucleus test. An interesting approach has been reported using a commercial reconstructed epidermis model, where the genotoxicity of chemicals has been tested employing the micronucleous assay (Chen et al. 2021; Magdolenova et al. 2014).
- ***Skin irritation:***
  Skin irritation is mediated by innate immune responses, involving some cell types and expression of specific biomarkers. Different *in vitro* methods have been described to be useful in the evaluation of TiO_2 as irritant, and even some OECD guidelines have been developed to use *in vitro* approaches instead of animals (Samberg et al. 2010; Sanches et al. 2020).

When designing and conducting NM toxicity studies in *in vitro* skin models, it is important to carefully consider factors such as NM characterization, dosimetry, and the relevance of the model to the intended exposure scenario. Appropriate positive and negative controls, as well as thorough statistical analysis, are also crucial for ensuring the reliability and reproducibility of the results. Overall, *in vitro* skin models have become an invaluable tool for assessing the dermal toxicity of NMs. By providing information on a range of toxicological endpoints, these models can help identify potentially hazardous NMs and guide the development of safer nanomaterials. As the field of nanotoxicology continues to evolve, *in vitro* skin models will likely play an increasingly important role in the safety assessment of NMs (Warheit 2018).

4 Computational Structure-Based Approach for Skin Nanotoxicity Prediction

The two approaches for NPs hazard assessment are experiential toxicology (*in vitro* or *in vivo* biological experiments) and *in silico* approaches (computational studies). In silico toxicology applies computational techniques to analyze, simulate, visualize, and predict NMs/NPs toxicity as well as chemicals and drugs (Costa et al. 2023a; Enoch et al. 2008; Kalantari et al. 2021; Khanna et al. 2015; Thwala et al. 2022; Trott and Olson 2010). To accurately predict particle toxicity, computational

models must account for their physicochemical complexity, which requires comprehensive characterization, as well as diverse exposure routes. Several models of different complexity have been developed, they predominantly use statistical and machine learning (ML) algorithms to establish relationships between NPs physicochemical properties and their consequent biological effects. Frequently used ML algorithms include regression, decision trees, support vector machines, artificial neural networks, partial least squares, and principal component analysis. On the other hand, structure-based techniques such as molecular docking coupled with molecular dynamics (MD) is also employed (Forest 2022). Predictive techniques such as Nano-Quantitative-Structure-Toxicity Relationships (Nano-QSTR models) are exclusive to NPs since it requires both previous determination of the physicochemical characteristics of NMs such as size, shape, surface area, and solubility and a proper experimental determination of the relevant skin nanotoxicity output (see Fig. 7.5). Physiologically based pharmacokinetic (PBPK) modelling coupled with molecular dynamics simulations are also known to foster a deeper understanding of NMs behavior, while, grouping and read-across strategies have significantly contributed to clustering, categorization, and classification of the most relevant NM properties linked to skin nanotoxicity, even when limited data are available. It is important to note that, in this computational context, from the methodological point of view the Nano-QSTR stands out for its predictive versatility oriented to multiple skin nanotoxicity outputs. This makes them better aligned with *in vitro* skin strategies compared with their in-silico counterparts (*i.e.*, molecular docking, molecular dynamics, DFT methods). The latter is more focused on answering specific questions at the molecular level from a mechanistic perspective on the interaction of NM and relevant molecular targets for skin nanotoxicity. All cited computational

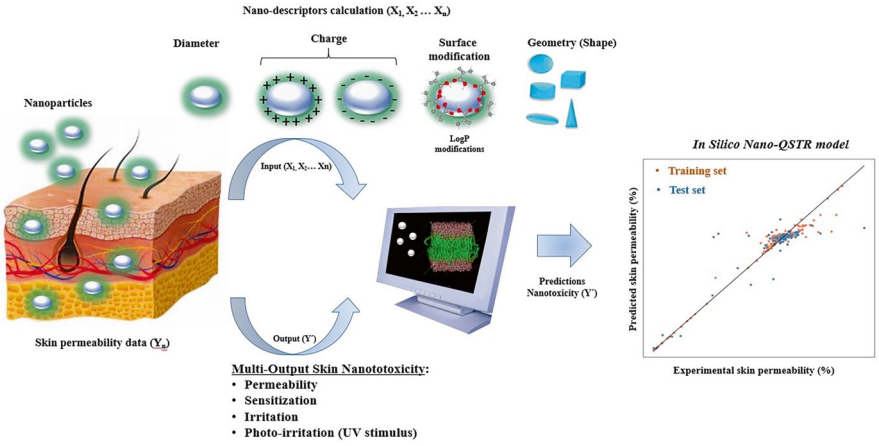

Fig. 7.5 Schematic diagram of a general workflow for in silico prediction of nanoparticle skin permeability (%) in humans by using a Nano-QSTR approach. Herein, nanodescriptors represent the physicochemical properties (e.g., diameter, charge, surface, and shape) of the NPs (from X1 to Xn) while the model output or predicted skin permeability (%) is denoted by the Yn

approaches are known to reduce the dependency on animal testing, saving time and resources as well as allowing a faster screening of larger numbers of NMs.

Recent advancements in computational modelling offer a promising approach for unveiling new insights into nanotoxicological evaluations, guiding risk assessment, and informed decision-making in nano-cosmeceuticals and skin health. By employing advanced in silico algorithms, researchers can anticipate potential adverse effects of NPs on skin permeability, sensitization, photo-induced irritation, as well as general irritation. Regarding skin sensitization of chemicals, several in silico models have been established (Toxtree, PredSkin, OECD's QSAR Toolbox, UL's REACHAcross™, Danish QSAR Database, TIMES-SS, and Lhasa Limited's Derek Nexus). These models utilize machine learning techniques and QSAR models to predict skin sensitization accurately, with some achieving correct classification rates of 70–80% on human data sets (Golden et al. 2021). Dermal permeation and absorption of substances have been also explored by in silico models, where mathematical equations can estimate the permeability coefficient of chemicals across the skin, taking into consideration features like skin anatomy, but also the physicochemical properties of the compounds to predict both local and systemic bioavailability applied to the skin, aiding in formulation risk assessment (Patel et al. 2022).

Quasi-QSAR was already employed to predict human keratinocyte cells (HaCaT) and human bronchial epithelial cells (BEAS-2B) cell viability when exposed to different 20 metal oxide nanomaterials. Hierarchical cluster analysis (HCA) and min-max normalization methods were employed in allocating codes for numerical descriptors (*e.g.,* core size, hydrodynamic size, surface charge, and dose). The established model provided good statistical and predictive performance (Choi et al. 2019). A new in silico model called Computational Indicator of Nanotoxicity exploits free energy analysis coupled with molecular dynamics simulations to evaluate the cytotoxicity of 2D nanomaterials can be relevant for skin toxicity evaluations (Tsukanov et al. 2022).

From the mechanistic point of view, several in silico strategies can be proposed, which to the best of our knowledge remain unexplored and represent a current gap in the field. The modelling of skin and epidermis nanotoxicity is directly influenced by critical NM physicochemical descriptors such as NPs size/diameter and shape which are recognized to have more weight during the design of computational models. For example, we could efficiently model the impact of the surface charge and/or coating of different NPs on the skin toxicodynamics by using structure-based molecular docking approaches (Lian et al. 2008) However, it is still challenging to implement the same modelling strategy for inorganic NPs since the mathematical scoring function to predict the thermodynamics binding affinity between NPs surface and the skin target receptors fails, when we want to model the influence of the different crystallographic planes of NP inorganic surfaces which significantly impact in the skin nanotoxicity (Norioka et al. 2021). To solve this issue, computational simulations based on Density Functional Theory (DFT) could be more successful in describing the influence of surface reactivity-based crystallographic planes for inorganic NPs. For example, DFT can simulate the TiO_2NPs anatase

crystallographic facets (101) which are highly stable, and its stability could lead to prolonged persistence in the human skin, potentially inducing oxidative stress and skin inflammation depending on the exposure time. In the case of anatase, the DFT modelling of the (001) plane could be more relevant to explain potential skin cytotoxicity-based interactions because this plane is known for its high reactivity (Kang et al. 2023; McLean and Zhan 2022; Tsukanov et al. 2022; Wilm et al. 2018).

To computationally predict the influence of these structural determinants together with the concentration and exposure time on the skin nanotoxicity; one could follow these methodological steps: (i) collect the existing experimental or theoretical data on NPs properties from public repositories or specific databases and simultaneously perform physicochemical nano descriptors calculation (as model inputs), (ii) data collection on toxicological skin effects (*i.e.,* irritation, photo-irritation, inflammation, sensitization, skin permeability, cytotoxicity, etc.), (iii) divide the dataset into different subsets, typically training (containing the 70% of the total dataset) and test sets (containing the 30% of the total dataset). Step (iv) performs a single or integrated multi-target output as the predictive Nano-QSTR model with machine learning procedure by implementing appropriate predictive algorithms using Python or state-of-the-art visual programming Knime-based workflow pipeline. (v) choose the best predictive model with the most relevant structural nano descriptors for skin nanotoxicity and statistical performance-based metrics (sensitivity and specificity), and (vi) model validation with an ad-hoc external dataset or experimental validation, when possible. This in silico strategy can help identify potential nano risks in skin-related applications.

5 Conclusion Remarks and Future Trends

Currently available skin models specifically developed to test nanomaterials and nanoparticles are scarce, indicating that this field is in its infancy. 2D, 3D static and dynamic models have been developed with technologies allowing precise control of cells placement creating complex skin structures that recreate the multiple layers of the dermis and epidermis and allowing researchers to conduct longer-term studies and gain a deeper understanding of NP's safety assessment. The design of new models for risk assessment purposes should consider: (i) the inclusion of hair follicles since it can support the evaluation of alternative routes of NPs absorption and excretion, (ii) tissue vascularization to allow the study of systemic exposure of dermally absorbed NPs, (iii) the inclusion of immune cells to grant to the model immune competency and (iv) tissue innervation with neural cells to allow the study of sensory reactions upon NMs exposure.

Computational modelling for predicting nanotoxicity has experienced significant advancements, however in the field of skin there still exist a gap of knowledge. From our point of view computational methods offer a promising alternative to traditional animal-based testing with possible high acceptance from the new approach methodologies regulatory context. Boosting machine learning algorithms in conjunction with extensive human data repositories, exhibit considerable promise

to predict skin permeability, sensitization, photo-irritation, and irritation induced by NPs/NMs. The continuous refinement and validation of these computational approaches are expected to lead to more precise nanotoxicity predictions and assist in the development of novel NPs with safer profiles for dermatological applications.

Acknowledgments This research was funded by the European Union's H2020 project Sinfonia (N.857253) and LEARN (N.101057510). SbDToolBox, with reference NORTE-01-0145-FEDER-000047, supported by Norte Portugal Regional Operational Programme (NORTE 2020), under the PORTUGAL 2020 Partnership Agreement, through the European Regional Development Fund. This publication is based upon work from COST Action European Network for Skin Engineering and Modeling (NETSKINMODELS), CA21108, supported by COST (European Cooperation in Science and Technology).

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