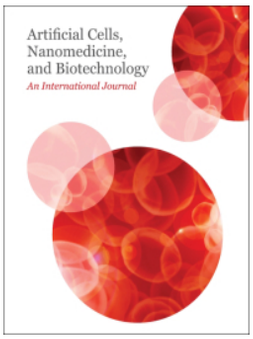

Taylor & Francis
Taylor & Francis Group

Artificial Cells, Nanomedicine, and Biotechnology
An International Journal

ISSN: 2169-1401 (Print) 2169-141X (Online) Journal homepage: www.tandfonline.com/journals/ianb20

In vitro 3D skin culture and its sustainability in toxicology: a narrative review

Syafira Masri, Mh Busra Fauzi, Nor Fadilah Rajab, Wing-Hin Lee, Diana Atiqah Zainal Abidin & Ee Ling Siew

To cite this article: Syafira Masri, Mh Busra Fauzi, Nor Fadilah Rajab, Wing-Hin Lee, Diana Atiqah Zainal Abidin & Ee Ling Siew (2024) In vitro 3D skin culture and its sustainability in toxicology: a narrative review, Artificial Cells, Nanomedicine, and Biotechnology, 52:1, 476-499, DOI: 10.1080/21691401.2024.2407617

To link to this article: https://doi.org/10.1080/21691401.2024.2407617

Published online: 03 Oct 2024.

ARTIFICIAL CELLS, NANOMEDICINE, AND BIOTECHNOLOGY
2024, VOL. 52, NO. 1, 476–499
https://doi.org/10.1080/21691401.2024.2407617

In vitro 3D skin culture and its sustainability in toxicology: a narrative review

Syafira Masri[a], Mh Busra Fauzi[a,b], Nor Fadilah Rajab[c], Wing-Hin Lee[d], Diana Atiqah Zainal Abidin[e] and Ee Ling Siew[e,f]

[a]Department of Tissue Engineering and Regenerative Medicine, Universiti Kebangsaan Malaysia, Cheras, Malaysia; [b]Advance Bioactive Materials-Cells (Adv-BioMaC) UKM Research Group, Universiti Kebangsaan Malaysia, Bangi, Malaysia; [c]Centre for Health Aging and Wellness, Faculty of Helath Sciences, Universiti Kebangsaan Malaysia, Kuala Lumpur, Malaysia; [d]Royal College of Medicine Perak, Universiti Kuala Lumpur (UniKL RCMP), Perak, Malaysia; [e]ASASIpintar Unit, Pusat PERMATA@Pintar Negara, Universiti Kebangsaan Malaysia, Bangi, Malaysia; [f]Faculty of Health Sciences, Universiti Kebangsaan Malaysia, Kuala Lumpur, Malaysia

ABSTRACT

In current toxicological research, 2D cell cultures and animal models are well- accepted and commonly employed methods. However, these approaches have many drawbacks and are distant from the actual environment in human. To embrace this, great efforts have been made to provide alternative methods for non-animal skin models in toxicology studies with the need for more mechanistically informative methods. This review focuses on the current state of knowledge regarding the *in vitro* 3D skin model methods, with different functional states that correspond to the sustainability in the field of toxicology testing. We discuss existing toxicology testing methods using *in vitro* 3D skin models which provide a better understanding of the testing requirements that are needed. The challenges and future landscape in using the *in vitro* 3D skin models in toxicology testing are also discussed. We are confident that the *in vitro* 3D skin models application may become an important tool in toxicology in the context of risk assessment.

ARTICLE HISTORY
Received 15 April 2024
Revised 3 September 2024
Accepted 11 September 2024

KEYWORDS
Alternative; non-animal; 3D skin model; toxicology; sustainability; vitro testing

Highlight points

This review on *in vitro* 3D skin models in toxicology highlights the urgent need for alternatives to traditional 2D cell cultures and animal models, which have significant drawbacks. Research is increasingly focused on developing 3D skin models that more accurately mimic human skin, thereby enhancing sustainability in toxicology testing. These models offer improved representation of human skin structure and function, facilitating deeper insights into the mechanisms of skin toxicity crucial for risk assessment. However, challenges remain, including technical limitations such as insufficient metabolic activity and the need for diverse cell types, alongside the necessity for rigorous validation to gain regulatory acceptance. Future directions include integrating advanced technologies like 3D bioprinting and microfluidics to enhance model complexity, as well as fostering collaboration among researchers, regulatory bodies and industry stakeholders to refine these models and establish them as standard practices in toxicology testing.

Introduction

The skin serves as the primary defence mechanism, protecting internal organs from the external environment, and enduring continual exposure to chemical, physical and biological threats [1]. The skin consists of two layers: the epidermis and dermis. The epidermis, which is the outermost layer of the skin, is composed of densely packed sheets of keratinocytes. These cells serve as a protective barrier against the external environment and regulate the loss of water [2]. Generally, the epidermis layer is densely packed with keratinocytes, which protect the skin from external infections, whereas the dermis layer serves as the skin's appendages, with fewer cellular elements, principally fibroblasts [3]. The dermis layer is surrounded by a complex connective tissue structure that contains neurons, hair follicles, glands and blood vessels used for nutrition transfer. Important sensations like touch, heat, pain and itching are transmitted by the extensive innervation that exists in the skin. Minimizing adverse reactions from treatments is crucial for maintaining the skin's physical barrier for protection, thermoregulation, sensibility, homeostasis and immunity. Adverse events affecting the skin can predominantly manifest from two sources: (1) direct contact with exogenous factors, such as therapeutics applied topically, or (2) indirect consequences, including side effects associated with systemically administered therapeutics [4]. The potential hazard illustrated in Figure 1 has the

CONTACT Ee Ling Siew sieweeling@ukm.edu.my ASASIpintar Unit, Pusat PERMATA@Pintar Negara, Universiti Kebangsaan Malaysia, Bangi 43600, Selangor, Malaysia

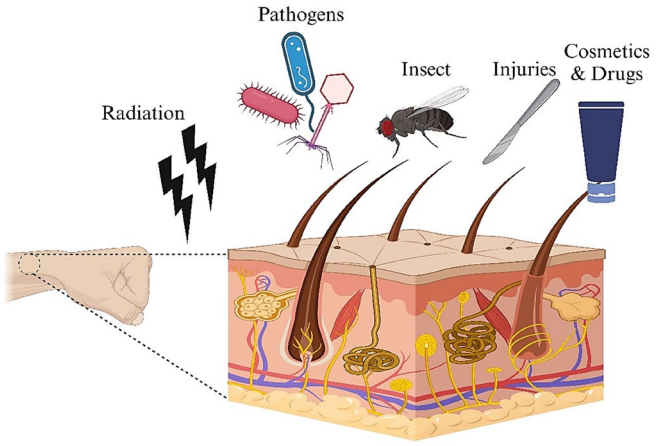

Figure 1. Human skin physiology that composed of three layers (epidermis, dermis and hypodermis). The exposure of skin to the external stimuli including radiation, pathogens, insects, injuries, cosmetics and drugs. Image created using Biorender.com.

capacity to induce cutaneous injury and toxicity in individuals who are exposed to the hazardous substances and substances.

To examine the potential toxicity of chemicals, medicines, or other compounds, skin testing is a standard procedure in toxicology. Skin toxicity testing can be done *in vivo* or *in vitro*. Toxicology testing is conducted to evaluate the potential harmful impacts that a chemical may have on a human and the surrounding environment [5]. A wide variety of chemicals exist, encompassing active pharmaceutical ingredients, cosmetics, as well as chemicals utilized in industry and household products [6]. Moreover, to screen compounds for possible harmful effects on people, household pets, and agricultural animals, validated *in vitro* models and animal experiments are commonly employed. Nevertheless, ethical considerations and the demand for improved efficiency and cost-effectiveness have spurred the advancement of new *in vitro* approaches for evaluating cutaneous toxicity. Moreover, the usage of animal models have drawbacks such as high cost, restricted treatments per animal, ethical problems in certain countries, and species variance, requiring supporting evidence from other animal models [7]. Over the years, studies of *in vitro* skin toxicity frequently make use of three-dimensional (3D) skin culture models rather than the conventional two-dimensional (2D) cell cultures since these models more accurately simulate the complicated structure of real skin.

Over time, there has been growing usage of *in vitro* skin toxicity testing, drive both by researchers and regulatory authorities seeking for alternative approaches to conventional animal testing. The anticipated revenue of the worldwide *in vitro* toxicity testing market was $10.8 billion in 2023 and is projected to reach $17.1 billion by 2028, with a compound annual growth rate (CAGR) of 9.5% from 2023 to 2028 [8]. Several examples of *in vitro* 3D skin models that are commercially available, including Labskin, Episkin™, EpiDermFT™ and MelanoDerm™, with varying properties and capabilities that demonstrate fair resemblance to native skin [9,10]. These models have been developed to aid in the assessment of phototoxicity, corrosivity and irritancy, as well as diagnostic testing. Researchers suggested that utilizing reconstructed human epidermis (RHE) kits is the most promising alternative to animal experiments [11]. Layers of cultured human cells are deposited onto a polymer matrix to create a skin reconstruction model. This allows for the inclusion of various cell types to achieve the appropriate composition and structural complexity. Most *in vitro* reconstructions include either a single epidermal layer or a full-thickness human skin equivalent (SE) [12].

SE and RHE exhibit considerable potential as substitutes for conventional *in vitro* assays. In addition, reconstructed human skin (RHS) has proven to be a valuable *in vitro* model for conducting toxicity tests on pharmaceuticals, chemicals and innovative materials. The OECD guideline 428 explicitly states that an *in vitro* human skin equivalent may accurately replicate the manufacture of several types of human skin, such as full-thickness skin, split-thickness skin and heat-separated human epidermis [13]. Further testing should closely resemble original skin since the quality of rebuilt 3D skin should be equivalent to human skin.

The advancement from 2D to 3D in vitro skin model

Two-dimensional (2D) cell studies were first used in the early 1900s and continued to be the preferred technique for the next century [14]. There is a notable development in the toxicology area from two-dimensional to three-dimensional (3D) *in vitro* skin models for toxicity assessment. Generally, utilizing a 2D cell culture is a convenient method for conducting biochemical experiments. The most frequent way for determining the level of toxicity *in vitro* is by the utilization of two-dimensional monolayer cultures that are composed of immortalized cell lines, stem cells and primary cells [15]. The 2D culture condition differs from the *in vivo* microenvironment, resulting in limitations such as lack of cell-cell and cell-extracellular matrix (ECM) interaction, loss of specific morphology/function/metabolic activity, and inability to replicate complex physiological and pathological environments or simulate heterogeneous diseases [16].

The benefits of 2D cultures are easy and cheap for functional testing. As a consequence, conventional 2D cell culture models often fall short in reproducing the *in vivo* skin microenvironment, thereby offering restricted insights into the physiological reactions of organisms to external stimuli [17]. The results obtained from isolating cells from their usual dynamic environment and growing them in stiff, static petri dishes are generally limited to cytotoxicity studies and lack the predictive power to explain drug efficacy in clinical settings. Thus, tissue engineering has enabled the development of an *in vitro* 3D skin model for toxicity assessment, serving as an alternative for the traditional 2D testing method.

Nowadays, advance techniques generally enable the attainment of greater model complexity, which subsequently leads to improved biomimicry to human native skin tissue [18]. To make reconstructed 3D skin models (epidermis models), epidermal keratinocytes are exposed to the air-liquid contact (with the help of trans well plates) or growth factors, which help the cells differentiate and form a layered epidermis that looks like the real skin tissue [19]. Furthermore, the epidermal layer can be cultivated on top of either a cellular or acellular dermis equivalent.

To construct *in vitro* models of human skin, several methodologies and techniques have been adopted from traditional cell culture, tissue engineering, 3D bioprinting and microfluidics. The 3D-bioprinting, also known as cell printing, operates on the same principles as 3D-printing. It involves the extrusion of cell suspensions in a bioink layer-by-layer in order to create multilayer tissue constructs [20]. Since live cells are so sensitive, printing cells and biological matter is very hard and complicated. The chosen of the materials, growth factors, differentiation factors, and cell types are very crucial for 3D-bioprinting.

In conventional manual inoculation methods, such as layering or mixing different cell types within a scaffold, there are limitations to the number of cell types that can be effectively incorporated into *in vitro* 3D skin models. Typically, these models mainly consist of keratinocytes and fibroblasts, which are the two primary cell types found in the skin's epidermis and dermis, respectively. These models aim to mimic the basic structure and function of human skin for various applications, including drug testing, cosmetic development and wound healing research. [18]. Thus, the usage of 3D-bioprinting as a fabrication strategy enable the incorporation of various cell types, such as keratinocytes, fibroblasts and adipocytes, to construct a complete skin layer (epidermis, dermis and hypodermis layer). Furthermore, intricate formations, such as blood vessels, may be reproduced by printing, even though they would often pose a difficulty [21]. Many previous studies demonstrated that the microenvironment of the printed model more closely resembles in human [22]. It was hypothesized that this enhanced similarity could lead to more accurate predictions in experiments involving pharmaceuticals, cosmetics and general research.

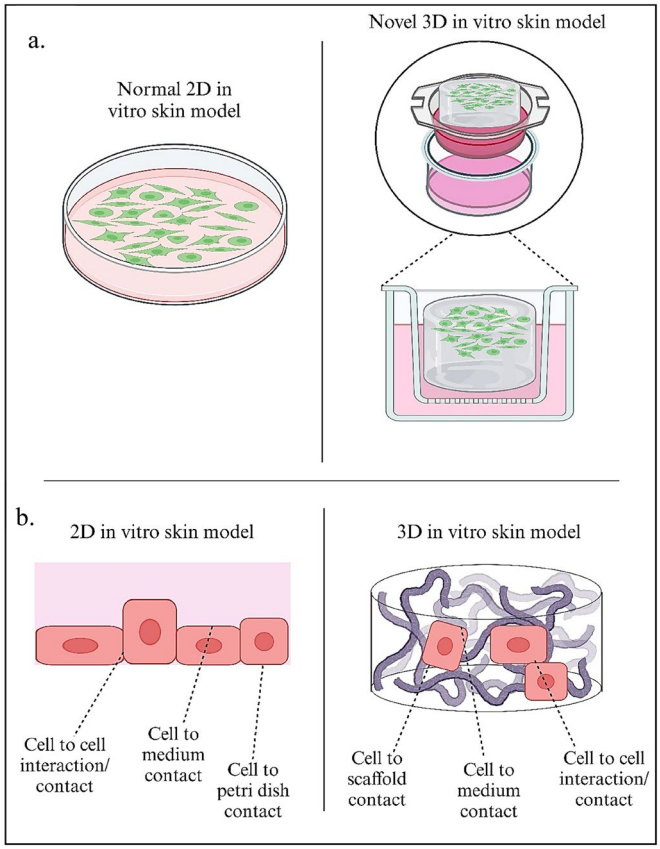

Figure 2. Illustration of culture models. (a) a comparison between normal 2D *in vitro* skin model that cultured in the normal petri dish and novel *in vitro* 3D skin model that fabricated *via* 3D-bioprinting and culture in air-lifting mode to mimic native human skin, (b) the interaction of cell-cell and cell-bioscaffold interaction between 2D vs *in vitro* 3D skin model. Image created using Biorender.com.

The 2D vs 3D culture models: are they significant?

In vitro skin models, whether 2D or 3D, exhibit differences in the interaction of cell-cell and cell-bioscaffold interactions due to their structural and organizational differences. In 2D models, cells are typically cultured on a flat surface, such as a tissue culture plate or slide. This configuration limits the ability of cells to interact in a three-dimensional manner (Figure 2a). Cell-cell interactions in 2D models primarily occur laterally across the cell membrane. While some degree of cell-cell communication and signalling can still occur, the spatial arrangement and organization of cells are limited. The lack of three-dimensional architecture in 2D models may not fully replicate the physiological cell-cell interactions found *in vivo*.

In 3D models, cells are cultured within a three-dimensional scaffold or matrix, allowing for more complex cell-cell interactions that better mimic the architecture of native tissue. Cells in 3D models can form multi-layered structures and establish more extensive cell-cell contacts, facilitating various cellular processes such as cell adhesion, migration and signalling. The three-dimensional arrangement of cells in 3D models enables the formation of cell-cell junctions, including tight junctions, adherent junctions and gap junctions, which play crucial roles in tissue integrity, barrier function and intercellular communication [14].

Figure 2b further shows that in 2D models, cells interact with the underlying substrate which serves as a support surface for cell attachment and growth. Cell-bioscaffold interactions in 2D models are primarily limited to the interface between the cells and the substrate. The properties of the substrate, such as its composition, stiffness and surface chemistry, can influence cell behaviour and phenotype. On the other hand, in 3D models, cells interact with the surrounding scaffold or extracellular matrix (ECM), which provides structural support and biochemical cues for cell behaviour. The interaction between cells and the scaffold in 3D models is more dynamic and multifaceted, influencing various cellular processes such as proliferation, differentiation and tissue remodelling. Cells in 3D models can remodel the surrounding ECM through processes such as matrix deposition, degradation and reorganization, leading to the formation of tissue-like structures and functional properties [23].

The *in vitro* 3D skin models better recapitulate the complex cell-cell and cell-bioscaffold interactions found in native skin tissue compared to 2D models. This enhanced mimicry of tissue architecture and microenvironment contributes to the physiological relevance and utility of 3D models in various applications, including toxicology.

In vitro 3D-bioprinted skin model

Recent developments in tissue biofabrication methods, such as the application of bioprinting technology, make it possible to generate biofabricated biological tissues in a repeatable

manner [24]. Research has demonstrated that bioprinting enables the precise development of three-dimensional stratified skin tissues in the form of multiwell plates [25]. The diverse bioprinting techniques allow for adding physiological complexity to replicate native human skin and build a more relevant chemical testing system [26]. With inherent spatial and chemical complexity, it can construct the reaction container, three-dimensional structure, endogenous cell-cell/cell-matrix interaction and *in vivo* microenvironment. There are several printing strategies and construct forms are universally applicable and correspond to various organ categories.

Briefly, 3D-bioprinting techniques are widely classified into different types based on the techniques used to deposit the bioinks including extrusion-based, jetting-based and vat photopolymerization. For jetting-based 3D-bioprinting, this technology uses controlled deposition of bioink droplets to form structures layer-by-layer. This category comprises a variety of approaches with its own mechanism for depositing droplets. Several methods may be used to achieve inkjet printing, such as thermal, piezoelectric and electro-hydrodynamic. Inkjet-based bioprinting entails the precise deposition of bioink particles onto the printing stage. Inkjet printing may be divided into two stages: firstly, the formation of discrete droplets that adhere to a specific region of the substrate; and secondly, the interaction between droplets and substrate [27]. Additionally, its multiple printing nozzles enable simultaneous printing of various cells, matrix materials and biological variables. As a result, this printing approach is being used for small-scale tissue/organ fabrication with high resolution needs [28].

To produce precise, repeatable and physiologically functioning constructions, bioink qualities, printer settings and substrate features must all be optimized. The jet break-up and droplet production in bioinks can be impacted by the presence of particles, such as cells. Long-term exposure to mechanical shear stress in biological cells can alter their phenotype and viability, and in the worst cases, cause cell lysis [29]. Thus, bioinks with optimum viscosity are typically easier to eject through the nozzle, reducing the risk of nozzle clogging. However, a previous study found that over 90% of the printed cells remained active after printing on the substrate [30]. Moreover, a previous study also has developed 3D skin tissue manufactured *via* inkjet printing using keratinocytes and fibroblasts to represent the epidermis and dermis, respectively, and collagen to constitute the dermal matrix [31]. Besides, a 3D skin tissue was produced by Lee et al. *via* inkjet printing multilayer constructs [31]. This study utilized both submerged and air-liquid interface (ALI) culture condition. After submerged media culture, 3D printed constructions were exposed to the ALI for epidermal layer development and stratification.

Although inkjet bioprinting is a common approach for generating complex biological structures by accurately depositing cells and biomaterials, however droplet formation and drop-on-demand (DOD) materials jetting will substantially influence the cell viability. DOD printing generates drops by propagating a pressure pulse in a fluid-filled chamber, typically through thermal expansion or piezoelectric actuation [32].

Currently, there are few studies exploring the impact of droplet impact on the cell viability of printed cells. Various models have been proposed to simulate cell viability post droplet impact, including the Newtonian model, a compound droplet model, and the ambient fluid model, where both cells and droplets are viscoelastic fluids with different properties [33,34]. Besides, the viability of printed cells within encapsulated droplets during DOD bioprinting is significantly influenced by droplet evaporation [35]. The droplet evaporation mechanism is a complex process influenced by fluid evaporation mode, physical parameters like temperature and pressure, solvent properties and interactions between solvent, particles and substrate. A previous study has investigated the effect of droplet performance of cell laden suspension found that as the bioink cell concentration increases, droplet size and velocity decrease, suppress satellite droplet formation and breakup time increases. Bioink has less ejected fluid volume, lower droplet velocity and longer breakup time compared to hard bead-laden suspension [32].

In the context of 3D hydrogel matrices used in the *in vitro* tissue engineering, the primary issue is the poor porosity of the hydrogels, which can impede the movement and dispersion of encapsulated cells [29]. Addressing the issue of low porosity in hydrogels is crucial for optimizing nutrition transport, waste elimination and cell migration in bioprinted structures. To address this limitation, the addition of natural polymers such as collagen, gelatine, hyaluronic acid and fibrin can improve the hydrogel's biological features and porosity.

Next, the extrusion mechanism in bioprinter provides improved structural integrity by continuously and precisely depositing filaments of high viscosity and cellular density with remarkable precision [36]. Extrusion-based bioprinting is a common approach in tissue engineering and regenerative medicine. The core mechanism entails mechanically pouring bioinks through a nozzle to produce the hydrogel. This dispersion equipment is popular owing to its ability to manipulate bioink aseptically and compatibility with a wide range of biomaterial viscosities [37]. However, mechanical force-induced shear stress may damage cells during extrusion-based bioprinting. This is dependent on the size of the needle or nozzle, the pressure, and the stiffness of the bioink. Every extrusion process involves shear stress, which should be considered in bioprinting. Thus, bioinks must have suitable rheological characteristics for bioprinting. For extrusion-based bioprinting, the most crucial parameter is the dispensing pressure. In order to force the ink out of the nozzle, there has to be enough pressure to overcome the surface tension of the ink. Hydrogels might not be forced to flow at low pressure, but they would jet under high pressure [38]. Numerous research has evaluated bioinks, which are a combination of cells and biomaterials, to enhance printability. Twelve different forms of hydrogels, such as collagen, chitosan, fibrin and alginate, were examined.

Besides, cell viability is another important aspect that must be considered. Shear stress significantly impacts cell biology, enhancing cell maturation and increasing stem cell differentiation. Dispensing pressure, nozzle diameter and viscosity will affect shear stress of the bioink, especially when the needle diameter is reduced in order to improve the printing resolution [39]. High pressure is required to extrude high viscous of bioinks, which can negatively impact cell viability. Moreover, a previous study found that dispensing pressure has a more significant impact on cell damage than nozzle diameter [40]. Besides, cell viability can be significantly influenced by factors

such as nozzle and chamber temperatures. The cell viability could be enhanced from 55.52% to 90% by regulating the nozzle and chamber temperature [41].

Nowadays, multiple studies have been carried out to develop biomimetic scaffolds in the extrusion-based bioprinting technique that maintain cell viability. A previous study by Yang et al. 2022 used extrusion-based 3D-bioprinting to construct an *in vitro* 3D human skin using human epidermal keratinocytes (HEKs) and human dermal fibroblasts (HDFs) using gelatine methacryloyl (GelMA) [42]. In this study, they used photo cross-linked to make cutaneous constructions. After 3 days of culture, HEKs were placed on top of the printed dermal layers and submerged to promote HDFs and HEKs growth. Moreover, they also utilized ALI culturing method to mimic the epidermal layer of skin. Moreover, a previous study by Jin et al. 2023 have developed a strong and elastin GelMA skin model using extrusion-based bioprinting [43]. Despite the adverse effects of the bioprinting procedure on cell survival, the majority of cells in the functional scaffold matrix (FSM) remained alive and after 7 days of cultivation, the epidermis and dermis of the FSM were clearly distinguished. Furthermore, a prior investigation conducted by Quı'lez et al. in 2020 has emphasized the necessary steps for preparing both the epidermis and dermis layers of the skin construct [44]. The skin models were incubated for 17 days to allow maturation process. Figure 3 below demonstrated the 3D *in vitro* model that successfully cultured by a previous study [45,46].

However, in the extrusion-based bioprinting, improving cell survival may depend on optimizing the formulation of the bioink. Moreover, the design and selection of optimal bioinks is a crucial phase in the bioprinting process. In order to have effective production of complex biomimetic tissue structures, bioinks need to have the following qualities: viscoelasticity, high mechanical integrity, adequate degradability, nontoxicity and non-immunogenicity One of the hardest parts of the bioprinting process is ink development. Nozzle design is crucial for bioink printability and cell survival, considering factors like bioink viscosity, shear thinning property and shear stress. Shear stress is a major cause of cell damage and death, and is directly proportional to the nozzle's inlet pressure. Increased pressure leads to increased shear stress for cells. Nozzle diameters in 3D-bioprinting range from a few micrometres to hundreds, with sizes generally used in cell printing ranging from 50 µm to 300 µm. Meanwhile, fibroblast cells, found in connective tissue, are typically 10 µm to 30 µm in diameter. Thus, in order to preserve the intended resolution and accuracy of the printed structure, the nozzle size needs to be carefully chosen so that cells pass through without being damaged.

Since a highly viscous bioinks can damage cells due to high shear stresses, so lower viscosity bioinks are used to prevent damage. More resolute and tuneable 3D printed structures can be created by creating agarose blends or chemically modifying agarose through carboxylation [47]. However, to improve cell adhesion and biological activity, agarose must be modified with bioactive groups or combined with other polymers like collagen or fibrinogen. Other than that, the shear-thinning behaviour of alginate may be adjusted by varying the concentration of the polymer. During injection bioprinting processes, alginate provides mechanical protection to cells, protecting them from damage caused by extensional flow [48]. High molecular weight (MW) of alginates are often added to bioink to enhance construct stiffness, but this can increase viscosity, making processing difficult and potentially damaging cells mixed with the polymer due to high shear forces needed during mixing or injection [49]. Thus, the issue can be resolved by manipulating the MW and MW distribution, specifically by combining high-MW and low-MW alginates. Furthermore, gelatine is combined with other polymers such as alginate to boost cell adhesion and viscoelastic properties of bioink, while hyaluronic acid is blended with fibrin to increase bioink printability [48,50].

Next, light-based vat-polymerization bioprinting is an advanced technique utilized in tissue engineering and regenerative medicine to develop 3D models. The method involves layer-by-layer polymerization of photosensitive bioinks in a vat using light to form a solid structure. Stereolithography (SLA) and digital light processing (DLP) are the primary methods used in light-based vat-polymerization bioprinting. Vat-polymerization bioprinters are categorized by their operation modes, which include single spot or plane photocrosslinking, linear or rotational patterning and light projection. The SLA system uses two methods to cure photo-curable bio-resin: top-down printing and bottom-up printing. Top-down involves a scanning laser above the vat, causing a resin layer to be cured and lowered into the vat for repeated curing. Bottom-up uses a scanning laser at the bottom of the vat, raising the build platform above the bio-resin vat [51]. A computer-controlled laser beam scans the photo-curable bio-resin surface to cure it to a pre-defined depth, adhering to the previous layer. The build platform is raised to recoate liquid photo-curable bio-resin and repeat the curing process for 3D complex constructs. The applied radial density should exceed the threshold, and excessive light exposure can cause bio-resin shrinkage and poor print accuracy.

The nozzle-free mechanism of this bioprinting method minimizes shear stress on living cells, unlike inkjet or extrusion-based bioprinting, with the main damage resulting from radiative stress from the light source and photoinitiator cytotoxicity. Thermal and radiative stress in cells is influenced by the light source. UVA, with a wavelength of 320–400 nm, can damage cells' nuclear DNA, leading to genomic mutations. UVB, with a wavelength of 290–320 nm, can induce cell apoptosis by activating the death receptor CD95 [52]. Thus, selecting visible light like blue light with a wavelength of 405 nm has a less severe effect on cell damage compared to UV light [53]. Next, lithium phenyl-2,4,6-trimethyl-benzoyl phosphinate LAP was found to be a more biocompatible photoinitiator than Irgacure 2959, especially at higher concentrations. The cell viability at 1-h printing time was 53% for 0.9% LAP, compared to almost zero for Irgacure 2959. The cell viability will decrease as the curing process increases. Figure 4 demonstrated the illustration images of different types of 3D-bioprinting technique that may influence cell viability during printing process. Moreover, Table 1 has summarized the fabrication techniques to develop *in vitro* 3D skin models.

Physiological properties of in vitro 3D skin model

The main goal of an *in vitro* 3D skin model is to reproduce the physiological attributes of authentic human skin more precisely in comparison to conventional 2D models. The emergence of

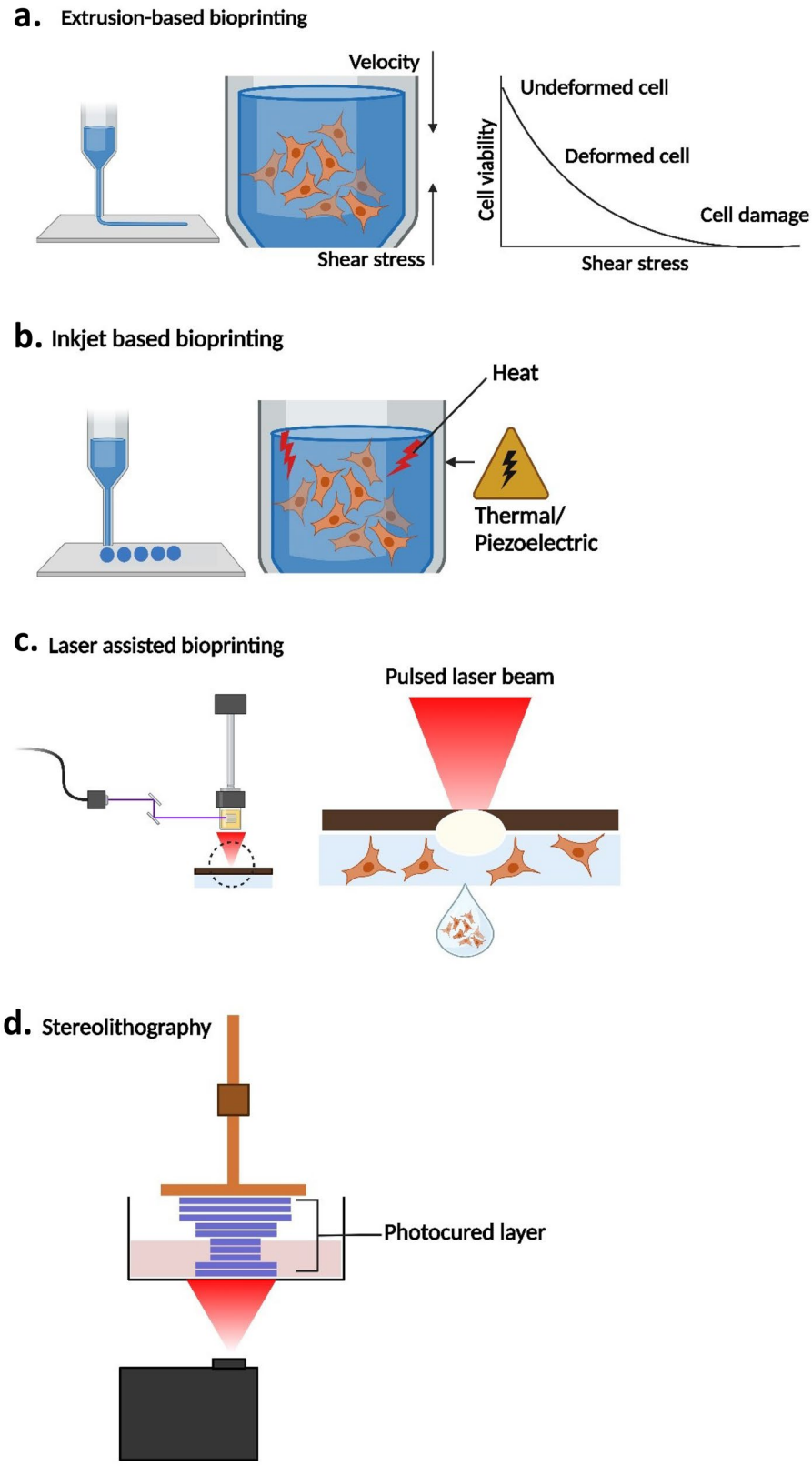

Figure 3. Gross appearance of *in vitro* 3D skin model (a) *in vitro* 3D skin model fabricated using gelatine-polyvinyl alcohol (PVA) bioink, both non-crosslinked and crosslinked with genipin extruded using extrusion-based bioprinting [45], (b) construction of three layers of *in vitro* 3D skin model and advanced skin model using collagen hydrogels [46]. used under the creative commons license (http://creativecommons.org/licenses/by/4.0/ accessed on 31 july 2024).

3D-bioprinting holds promise for a transformative change in the domain of skin tissue engineering through the automated production of human skin equivalents that are both physiologically significant and structurally complex.

A 3D-bioprinted *in vitro* human skin model holds substantial promise for assessing cosmetics, medications and gaining insights into the complex physiological mechanisms critical to human skin. This would help eliminate the disparity between

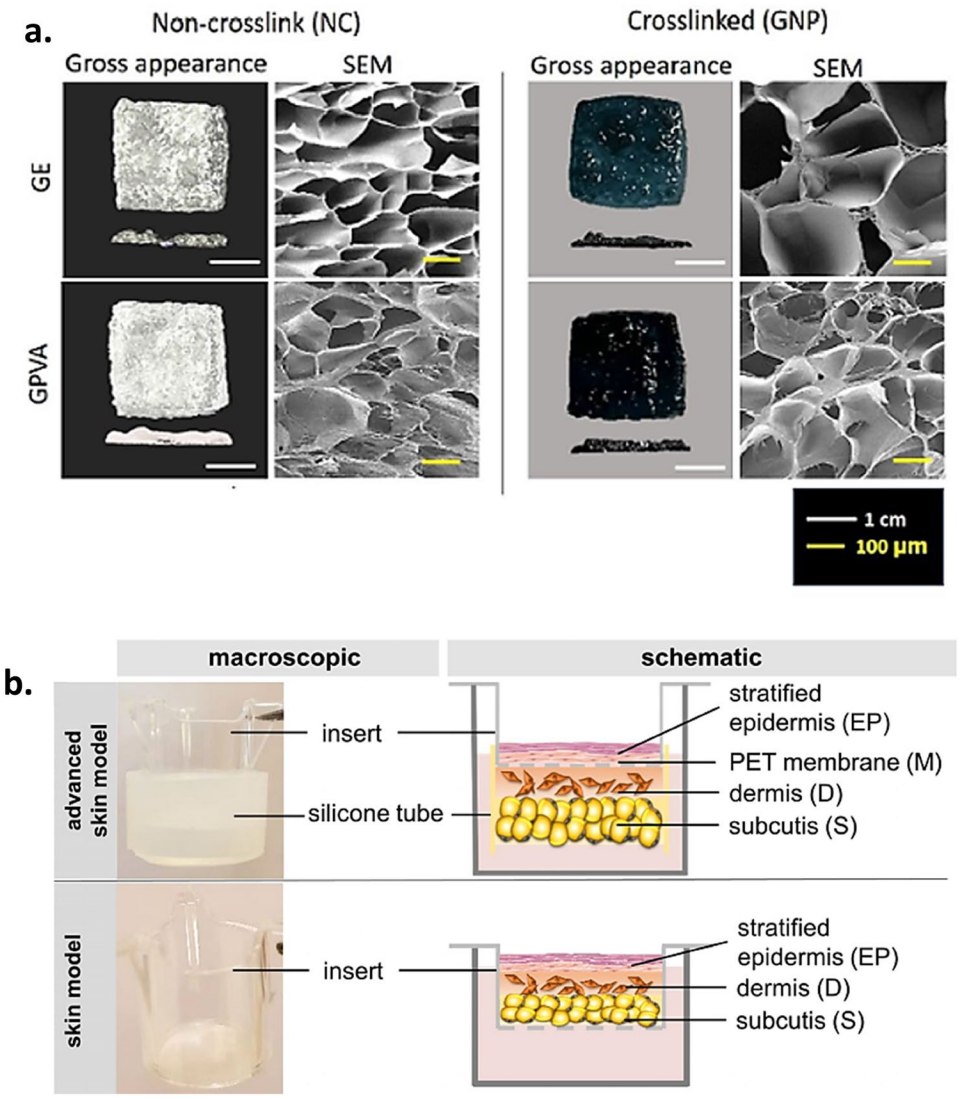

Figure 4. Illustration images of different type of 3D-bioprinting techniques (a: extrusion-based, b: inkjet-based, c: laser-assisted and d: stereolithography) and how these printing techniques may affect cell viability during printing process. Image created using biorender.com.

Table 1. Summarization of fabrication technique of *in vitro* 3D skin models.

Type of 3D-bioprinting	Skin layer	Cell types	Bioinks	Condition	Efficacy	References
Inkjet-based bioprinting	Epidermis and dermis	Keratinocytes and fibroblasts	Collagen	After 7 days of growth under submerged conditions, printed skin constructions were developed for 7–14 days at the ALI. The thickness of the skin constructions decreased drastically within the first 24 to 48 to *500 mm and continued to shrink steadily throughout the culture period. Histology and immunofluorescence analysis showed that 3D printed skin tissue closely resembled human skin tissue *in vivo*.	Demonstrates precise control over the deposition of cells and biomaterials, facilitating the creation of complex tissue models for accurate evaluation of toxic effects.	[31, 54]

(*Continued*)

Table 1. Continued.

Type of 3D-bioprinting	Skin layer	Cell types	Bioinks	Condition	Efficacy	References
Extrusion-based bioprinting	Epidermis and dermis	Keratinocytes and fibroblasts	Gelatine methacryloyl (GelMA-rhCol3)	Higher rhCol3 concentrations increase cell proliferation, leading to greater confluent (~100%) spreading of epidermal keratinocytes at an early stage (3 days) compared to rhCol3-free counterparts. Approximately 40 µm thick epidermis layer were successfully printed out *via* 3D-bioprinting.	Its capability to deposit bioinks containing multiple cell types and biomaterials in a layer-by-layer fashion, enabling the creation of complex tissue models that closely mimic human physiology for accurate evaluation of toxicological responses.	[42]
Extrusion-based bioprinting	Epidermis and dermis	Keratinocytes and fibroblasts	Gelatine methacryloyl (GelMA)	*In vitro*, 3D-bioprinting functional skin model (FSM) maintained a moist microenvironment and barrier function and mimicked the normal skin environment to enhance cell survival and proliferation. Moreover, FSM is capable of maintaining cell viability for at least 1 week.		[26]
Extrusion-based bioprinting	Epidermis and dermis	Keratinocytes and fibroblasts	Mixture of bioinks: hFbs: 20.000 cell/ml. Fibrinogen: 1.2 mg/ml. CaCl2: 8% v/v. Amchafibrin: 0.8% v/v.	Printed skin resembles natural human skin and is difficult to differentiate from bilayered dermo-epidermal counterparts.		[44]
Laser-assisted bioprinting (LaBP)	Epidermis and dermis	Keratinocytes and fibroblasts	Alginate and 50% ethylenediaminetetraacetic acid (EDTA) human blood plasma	This work offers promising results to generate 3D *in vitro* models and tissue replacements for various tissue engineering applications. The adherent and gap junction development were observed. It is crucial for tissue morphogenesis and cohesiveness. This work demonstrated an effectiveness of LaBP as a tool for creating complex 3D structures that closely resemble the functionalities of natural tissues.		[55]
Extrusion-based bioprinting	Dermis	Fibroblasts	Sodium salt of alginic acid (ALG), carboxymethyl cellulose (CMC), and calcium chloride	The findings indicated that the bioink including fibroblasts effectively sustains fibroblast cellular function for a duration of 29 days and exhibits satisfactory printing capabilities within a biologically suitable 3D setting. Consequently, this bioink has promise as a valuable resource for the fields of skin tissue engineering and drugs testing.		[56]

(*Continued*)

Table 1. Continued.

Type of 3D-bioprinting	Skin layer	Cell types	Bioinks	Condition	Efficacy	References
Pneumatic extrusion-based bioprinting	Epidermis	Laminin and keratinocytes	Gelatine, sodium alginate and fibrinogen	The printed constructs exhibited a sustained high level of cell viability for a minimum of 7 days following the printing process. Full-thickness skin was created by incorporating laminin solution and seeding epidermal cells followed by ALI culture that for drug screening, cosmetic testing and clinical treatment.		[57]

conventional monolayer or 3D cultures and animal models. Figure 2a above illustrated the different between cell-cell interaction in 2D vs 3D *in vitro* skin model. An *in vitro* model that integrates both skin and neurons would have significant utility across several research disciplines, including dermatology, neurosciences and toxicity assessment [58]. The incorporation of sensory neurons in a skin model, in particular, would allow for the investigation of skin sensitization while applying novel cream formulations or unravelling underlying disease causes. In dermatology practice and research, neuronal-triggered responses including pain, irritation, allergy, skin inflammation and regeneration are also significant [59].

An *in vitro* 3D skin model's physiological features are intended to mimic the complexity and functioning of original human skin. Keratinocytes constitute approximately 90% of the epidermis; these cells undergo keratinization and differentiation to produce a stratified epidermis [60]. Keratinocytes secrete cytokines that promote inflammation in reaction to environmental stimuli, including ultraviolet light, allergens, pathogens, pains and any sensitization. Generally, full-thickness human skin equivalents (FTSE) and reconstructed human epidermis (RHE) are two models that effectively replicate the attributes of the human epidermis [60]. The term "RHE" refers specifically to the epidermis, as it is composed only of keratinocytes that are cultivated at the interface between air and liquid. The FTSE contains both the outermost layer of skin, known as the epidermis, and the layer underneath it, called the dermis. As compared to RHE, FTSE involves seeding of keratinocytes onto dermal-like materials, hydrogel combined with fibroblasts, de-epidermized dermis (DED), or self-assembled dermal sheets in FTSE models. Generally, the RHE is commonly employed for conducting skin corrosion experiments [6]. The skin corrosion test assumes that corrosive agents can diffuse or erode through the stratum corneum and damage underlying cell layers. Corrosive chemicals decrease cell viability below defined thresholds (<50% after 3 min exposure – Class 1 A, ≥50% after 3 min exposure AND <15% after 60 min – Class 1B and 1 C), while noncorrosive chemicals.

In vitro 3D skin model for toxicity testing

In vitro 3D skin models are utilized to evaluate the toxicity of a wide range of products and chemicals [61]. There are many cosmetic products contain chemicals such as preservatives, fragrances, colourants and UV filters. These models are employed to assess the safety of these ingredients to ensure they do not cause adverse effects such as irritation, sensitization, or allergic reactions. Drugs and pharmaceutical compounds may also have topical applications, and their safety on the skin needs to be evaluated. The *in vitro* 3D skin models assist in assessing potential side effects, including irritation, inflammation and cytotoxicity, which can arise from drug formulations or their metabolites. There are increasing production of industrial chemicals, including solvents, surfactants, metals and pesticides, which in contact with the skin in occupational or environmental settings. *In vitro* 3D skin models are used to determine the potential dermal hazards of these chemicals, such as skin irritation, corrosion and absorption. Additionally, personal care products such as soaps, shampoos, lotions and deodorants contain numerous chemicals that may pose risks to skin health and *in vitro* skin models help evaluate the safety of these products by assessing their potential to cause irritation, sensitization, or other adverse reactions [46].

Pesticides, herbicides and fertilizers used in agriculture may come into contact with the skin during handling or application. *In vitro* 3D skin models are employed to evaluate the dermal toxicity of agrochemicals and assess their potential to cause skin irritation, sensitization, or systemic effects. The pollutants present in the environment, such as heavy metals, polycyclic aromatic hydrocarbons (PAHs), and volatile organic compounds (VOCs), affect skin health upon dermal exposure. Therefore, *in vitro* skin models are used to assess the toxic effects of environmental pollutants and identify potential mechanisms of skin damage.

The *in vitro* 3D skin models are valuable tools for assessing the toxicity of diverse chemicals, contributing to the development of safer products and regulatory decisions aimed at protecting human health. Commercial tissue manufacturing standardization, international validation and regulatory approval have led to the widespread usage of 3D reconstructed human skin models in industrial and academic research labs worldwide [62]. Skin safety testing that commonly being utilized are including, corrosiveness, irritation, phototoxicity and skin absorption. Meanwhile effectiveness tests include barrier, moisturizing, anti-inflammatory, UV protection and percutaneous absorption. Despite the fact that

many skin models are utilized for a variety of skin-related assays, including skin corrosion, skin irritation, skin barrier development, and skin absorption, epidermal models are the only ones that are permitted for use in *in vitro* testing [63].

Skin irritation

An *in vitro* 3D model with a hydrogel system is a potential way to test for skin irritation that aims to give more accurate and useful results than standard 2D cell cultures. The cosmetics and pharmaceutical industries utilize skin toxicity research to determine if novel substances are harmful to human skin. Skin irritation test is a chemical testing that can induce reversible skin injury, which is an inflammatory reaction that often resolves within a few days [64]. Generally, two processes may cause skin irritation: disruption to the stratum corneum barrier function and direct irritating effects on skin cells [6]. Previously, the *in vitro* reconstructed human epidermis (RHE) test technique used cell viability as a readout to assess cell/tissue damage.

For skin irritation testing, keratinocytes play a crucial role in controlling skin inflammatory reactions, and the production of extracellular IL-1α is considered an important indicator of chemical skin irritation [65]. Thus, according to a previous study, keratinocytes, dermal fibroblasts and endothelial cells have been incorporated into a microfluidic device to develop a novel skin-irritation platform utilized chemical irritants include sodium lauryl sulphate (SLS) and steartrimonium chloride (SC) [66]. Angiogenic sprouting is promoted by interactions between keratinocytes and dermal fibroblasts on the microfluidic device. Since SLS and SC are ubiquitous in cosmetics, their irritative effects are of special relevance. As sprout length increased, SLS greatly affected angiogenic performance. SC, despite being non-irritating and used in safe formulations, promoted vessel sprouting and lumen development at 10, 20 and 60 µM. Furthermore, Ma et al. 2021 constructed 3D skin models that were utilized to assess skin irritation from resident products and cleaning agents in a prior study [61]. The findings indicated that 0.3% SLS and 1% Triton X-100 in 3D skin models led to less than 20% tissue activity and elevated IL-1α release. A positive control for resident cosmetics is 0.3% SLS, while 1% Triton X-100 is recommended for cleaning products. After analyzing tissue activity and IL-1α levels, they identified that four cosmetic products potentially cause skin irritants.

To date, skin exposure to manufactured nanomaterials, including metal nanoparticles, nanostructured hydrogels, polymers and carbon-based nanomaterials, is a growing concern due to potential health risks. Thus, numerous studies have examined the effects of nanoparticles (NPs) on cutaneous irritation. Scientists from all over the world are now interested in nanotechnology because it could be used in many different areas, such as medicine, cosmetics, construction, food, sensing gases, electronics, medicine, diagnostics, medication delivery and more [67]. Many researchers highlighted that acute cutaneous irritation and corrosion testing of silver nanoparticles (AgNPs) in rabbits did not result in skin damage, including erythema, eschar, or edoema formation, for 72 h after exposure [68,69]. Several recent research utilized *in vitro* testing using cultivated skin-derived cell lines to investigate the skin corrosion/irritation caused by NPs. Although nanosilica NPs were toxic to human keratinocytes *in vitro*, they had no such effect in an *in vitro* 3D skin model [70]. Apart from that, AgNPs were found to be reduced the viability and elevated the levels of inflammatory cytokines, including interleukin (IL)-1β, IL-6, IL-8 and tumour necrosis factor-alpha (TNFα), in cultured human epidermal keratinocytes [71,72]. However, as compared to the *in vitro* model, titanium NPs (TNPs) and quantum dot NPs reduced the cell viability rate of cultured HaCaT human keratinocyte cell line survival [73,74].

In addition, skin irritation was assessed using the SkinEthic™ Reconstructed Human Epidermis, a three-dimensional epidermal tissue made up of normal human keratinocytes grown at the ALI [75]. According to the study, acute exposure to graphene-based materials (GBMs) containing minimal irritant surfactant residues does not result in skin irritation. However, to sustain stable dispersion of biologically designed GBMs, non-irritant exfoliants such as melamine are recommended. The GBMs with irritant surfactants (FLG-SDS and FLG-SDBS) reduced RhE viability at levels below 50%, indicating irritant properties.

Skin corrosion

Generally, skin corrosion tests have been conducted on animals, such as rabbits, following the OECD test guideline (TG 404) [76]. However, *in vitro* skin corrosion and irritation testing using animals has been replaced with *in vitro* skin models due to animal welfare concerns. EpiSkin™ and Epiderm™ models were approved by ECVAM in April 2007 to replace *in vivo* rabbit skin irritation testing. They are also utilized for regulatory purposes, including *in vitro* skin irritation (OECD TG 439) and skin corrosion (OECD TG 431) examinations of cosmetic chemicals [77]. Thus, *in vitro* data gathered from alternative approaches employing human skin models is being used to classify chemicals that cause skin corrosion and irritation. Commercially available 3D models of human skin, including EpiSkin™, EpiDerm™ and SkinEthnic™, are widely used for alternative skin corrosion and irritation tests [78].

The Chinese National Standard GB 30000 establishes 28 mandatory safety requirements for chemical categorization and labelling, each corresponding to a danger class under UN GHS (AQSIQ, GB 30000. 2 to GB 30000. 29, 2013). The Draize rabbit test (GB 30000.19) divides skin irritation and corrosion into three subcategories (1 A, 1B and 1 C), irritation (Category 2), and mild-irritation (optional Category 3) [79]. EpiSkin is an *in vitro* skin model that is created by culturing normal human keratinocytes on a collagen matrix [80].

The researchers utilize reconstructed human epidermis, derived from human-derived non-transformed epidermal keratinocytes, which closely resembles the properties of the upper parts of human skin. A previous study by Choi et al. 2014 examined the impact of nanoparticles and their combination on skin corrosion and irritation by utilizing the *in vitro* 3D human skin model, KeraSkin™ [78]. The cell viability

remained at its pre-determined threshold level of 15% after 60 min of exposure in the corrosion test and 50% after 45 min in the irritation test. Furthermore, the *in vivo* test conducted on rabbits also demonstrated outcomes that were non-corrosive and non-irritating. Next, the increasing use of graphene-related materials (GRMs) in electronics, healthcare and other fields requires careful assessment of their health effects. Skin contact is a major GRM exposure route. Thus, GRM skin exposure was assessed using an *in vitro* 3D reconstructed human epidermis (RhE) model. In this study, the latter were not irritants or corrosives after testing a broad panel of powdered GRMs.Only GRMs with unremoved irritating surfactants reduced RhE viability to levels below skin irritation (≤50%, after 42 min exposure and 42h recovery), but not below corrosion (<50%, after 3 min exposure or <15% after 1h exposure) [81]. However, while RHE models possess the benefit of encompassing all epidermal layers of the epidermis, they may occasionally fail to differentiate between the basale, spinosum and granulosum strata, which is a critical consideration in penetration studies.

Phototoxicity

Skin redness is linked to phototoxicity caused by chemicals that are absorbed by the skin and then exposed to ultraviolet light. It is important to determine if active chemicals that are applied on the skin can cause phototoxicity. Nowadays, the practice of decorative and cosmetic tattooing is common. The phototoxic impacts of tattoo pigments are subjects of concern for regulatory bodies, toxicologists and the pertinent industry sectors [82]. Tattoos can cause inflammatory, eczematous, photo-aggravated, granulomatous, lichenoid, and pseudo lymphomatous skin reactions. The two most frequent tattoo pigment hypersensitivity responses are allergic contact dermatitis and photoallergic dermatitis. Previous research tested the phototoxicity of six tattoo pigments: cadmium sulphide, carbazole, selenide, mercury (II) sulphide, chromium oxide and cobalt aluminate, using the 3T3 NRU PT and 3D human skin model [82]. According to the findings, cadmium sulphide and carbazole both have the potential to be phototoxic. Moreover, it was proven by the 3D human skin model that only carbazole was phototoxic.

Moreover, phototoxicity may be detected by employing a skin model to assess the potential toxicity of sunscreen formulations, both with and without exposure to UV light. Furthermore, the toxicity of these formulations was assessed following exposure to photodegradation [83]. The skin models' capacity to effectively measure UV radiation's effect on cell viability is shown by their persistence in PBS in dark and light settings. When applied topically, sunscreens were less harmful than PBS in all formulations/conditions, even the control formulation (BF). Long-term photodegradation by UV radiation can reduce toxicity and phototoxicity in sunscreen formulations compared to those not exposed to UV radiation or left in the dark.

Besides, the purpose of the EpiDerm™ H3D–PT assay is to determine the phototoxic potency and potential of formulations and compounds that are applied topically [84]. Due to the fact that the assay permits the application of test materials to an air-exposed surface (stratum corneum), it mimics the *in vivo* environment and may therefore enable the prediction of the phototoxic potency of usage concentrations of test materials.

Skin sensitization

A skin sensitiser is a chemical that causes an allergic reaction when it comes into contact with the skin, as described by the United Nations Globally Harmonized System of Classification and Labelling of Chemicals (UN GHS). An Adverse Outcome Pathway (AOP) is a conceptual construct that presents knowledge on the relationship between a molecular starting event and an undesirable outcome at a biological level relevant to risk assessment. The AOP starts with the molecular initiating event (MIE) and ends with the adverse outcome. The intermediate relationships between biological scales are determined by available mechanistic, causal, or associative information from *in vivo*, *in vitro*, or computational approaches [85]. The AOP summarizes the chemical and biological pathways of skin sensitization from molecular triggering event to allergic contact dermatitis [86].

Firstly, electrophilic compounds covalently attaching to nucleophilic sites in epidermal proteins is the initial molecular starting event. The second major event in this AOP occurs in keratinocytes and involves inflammatory responses and gene expression alterations related with antioxidant/electrophile response element (ARE)-dependent cell signalling pathways. The third essential event is dendritic cell (DC) activation, measured by cell surface markers, chemokines and cytokines. The mouse Local Lymph Node Assay indirectly measures T-cell activation and proliferation, the fourth essential event.

Skin sensitivity is a complicated process that begins with the xenobiotic getting through the skin [87]. The first step in sensitization is chemical penetration through the stratum corneum into the viable epidermis layers [88]. Most molecules that can pass through the skin barrier are too small to have an inherent sensitizing effect, and this effect often relies on how well they can form strong covalent bonds with proteins found naturally in the skin. Allergic contact dermatitis (ACD) arises from persistent skin contact with an allergen. The ACD activates the innate and adaptive immune systems through sensitization and challenge [64]. In the first step (induction), chemical allergen exposure produces skin sensitivity. If sensitized people are exposed to the same allergen or a cross-reactive allergen again, the elicitation phase begins. Thus, ACD prevention should be addressed, requiring substantial research into sensitization processes to identify sensitizers, forecast their potency, and most crucially, predict their safe (no reaction) concentrations.

Alternative approaches are incorporated into Adverse Outcome Pathways (AOP) and are based on the body of information that currently exists about the impacts of molecular perturbations at the subcellular, cellular, tissue, organ, whole animal and population levels. The objective of the *in vitro* 3D models is to simulate the complex interaction of cells and tissues that exists within the human epidermis. In order to

construct a layered epidermis, human keratinocytes (HEKs) are grown at the ALI and then reconstructed to make RhE or 3D epidermal models [64].

A previous study has created a novel *in vitro* skin sensitization assay utilizing reconstructed human epidermis (RhE) model [89]. This assay is anticipated to possess a wider range of applicability compared to current *in vitro* assays. After 6 h of treatment with skin sensitizers 1-fluoro-2,4-dinitrobenzene and oxazolone, and not benzalkonium chloride, microarray analysis showed significant upregulation of five genes (ATF3, DNAJB4, GCLM, HSPA6 and HSPH1) related to cellular stress response in the RhE model.

The Guinea Pig Maximization Test (GPMT) and the Buehler Test (TG 406) (OECD, 1992) are *in vivo* procedures that encompass the whole skin sensitization process [90]. In 2011, the local lymph node assay (LLNA: DA), which is an alternate way of refinement that makes use of mice, was incorporated into the standard. The LLNA: DA (TG 442 A) (OECD, 2010) has gained widespread acceptance because it evaluates just the induction response. This is because it has benefits over the guinea pig tests, both in terms of animal welfare and because it offers an objective evaluation of the induction stage of skin sensitization [91]. On the other hand, new alternatives to animal testing for skin sensitization potential classification in cosmetics and chemicals have raised the possibility that *in vitro* testing could be more efficient than *in vivo* testing in terms of time and money. Therefore, alternatives to conventional animal testing, *in vitro* models, and 3D models in particular, are becoming increasingly popular for determining skin sensitization. Registration, Evaluation, and Authorization of Chemicals (REACH) indicates that *in vivo* tests should only be done when *in vitro* or in chemico testing methods are ineffective or when the results of these tests are unsuitable for risk assessment and classification [92].

Genotoxicity

A genotoxicity study employing a 3D *in vitro* skin model aims to assess the ability of a chemical to induce genetic harm, such as DNA mutations, changes in chromosomes, or other disturbances in the genetic material, inside a human skin tissue structure. This methodology is of utmost importance in the field of toxicology since it allows for the evaluation of the safety of chemicals, cosmetics, medicines and other compounds without the need for animal experimentation. In order to evaluate the risk to human consumers, it is necessary to characterize the genotoxicity of a variety of biological and chemical constituents, including natural compounds, that are extensively employed in the pharmaceutical industry, daily necessities and cosmetics. Genotoxicity testing often involves the assessment of point mutations and chromosomal damage. The chromosomal aberration test, as outlined in the Organization for Economic Co-operation and Development Guideline for the testing of Chemicals (OECD), specifically guideline no. 473, and the micronucleus assay, as described in OECD guideline no. 487, are widely employed methods for studying chromosome damage [93].

Human reconstructed skin micronucleus (RSMN)

The genotoxicity hazard of a substance is typically assessed through *in vitro* assays that examine gene mutations and structural and numerical chromosomal aberrations. The *in vitro* micronucleus (MN) assay is a widely used method for detecting clastogenicity, offering a more efficient alternative to the CA assay due to its enhanced ability to detect aneugenic compounds. The assay employs 2D standard cell cultures, which have limitations in handling stress, resulting in a high percentage of MP results [94]. However, 3D tissue constructs are believed to be more *in vivo*-like and exhibit metabolic competency similar to native human skin. Thus, the RS Comet and MN assays were developed and validated as ideal building blocks for a replacement of tier 2 animal follow-up assays for dermal exposure.

Generally, EpiDerm™ *in vitro* human reconstructed skin micronucleus (RSMN) test is a promising method for determining the genotoxicity of topically applied substances. Thus, the Cosmetics Europe Genotoxicity Task Force coordinates and funds validation studies for the reconstructed skin micronucleus assay (RSMN) using the Epiderm™ model [95]. The historical data were examined, indicating that vehicle control and positive control values for percentage micronuclei in binucleated cells are within previously reported thresholds. Technical issues, such as the evaluation of several solvents with 48 and 72-h treatment regimens, are being studied. In addition, RSMN assay in MatTek Epi-200™ skin models also have been validated and 43 coded compounds selected by independent experts were tested in four US/European laboratories [96]. The RSMN assay demonstrated low background micronucleus frequency and pro-mutagen metabolism capacity, with 80% accuracy compared to *in vivo* genotoxicity outcomes, 75% sensitivity, 84% specificity, and 77–80% reproducibility between and within laboratories.

3D skin comet assay

Generally, comet assay may detect both chromosomal damage and DNA lesions that may cause gene mutations, complementing the RSMN detection of chromosomal damage. The 3D Skin Comet assay detects strand breaks to assess DNA damage, providing insights into genetic damage and DNA integrity. It uses 3D models to mimic human skin architecture, enabling accurate simulation of dermal exposure scenarios. In 2006, SkinEthic™ published the first study involving test compounds applied to a 3D reconstructed skin model, EpiSkin™ [97]. The study evaluated DNA damage in dendritic cells cultured in a medium below the skin model, rather than skin cells. Previously, the comet assay was adapted to EpiDerm™- and Phenion® Full Thickness Skin Models, and tissue-specific protocols for single cell isolation and the general comet assay were transferred to European and US-American laboratories [98]. The 3D Skin Comet assay demonstrated high predictive capacity and good reproducibility, with four laboratories reaching 100% predictivity and the fifth yielding 70%.

Moreover, the Phenion® Full-Thickness Skin Model was used for inter-laboratory validation of the 3D Skin Comet assay in order to assess its potential as an *in vitro* tool for

monitoring positive findings from standard genotoxicity testing [99]. The skin model demonstrated similar metabolic competency to natural human skin, with high predictive capacity for 32 tested substances, with a sensitivity of 80%, specificity of 97% and overall accuracy of 92% compared to *in vivo* animal genotoxicity test outcomes.

Ideal physical & mechanical properties of *in vitro* 3D skin model in toxicity testing

A n *in vitro* 3D skin model is an artificial system that mimics human skin structure and function in a laboratory setting, used for research and testing in skin biology, drug delivery, toxicity, and other applications are requiring accurate physicochemical properties. In tissue engineering field, different biomaterials have been used to develop *in vitro* 3D skin models. Natural-based bioinks, such as alginate, gelatine and collagen, provide superior biocompatibility, accelerated biodegradation rate, absence of toxicity and optimal mechanical stability. Predictable testing is urgently required for the preclinical development of new skin therapies. These testing setups serve as the basis for evaluating the effectiveness, absorption and safety of the skin therapeutics. Thus, the optimum characteristics for the production of *in vitro* 3D skin models are summarized in Table 2.

Structural similarity of human skin equivalent with native human skin

The epidermis, dermis and hypodermis are the three separate layers that made up the skin. Each layer interacts with the others in a synergistic manner in order to keep the cutaneous health and function in good condition. When it comes to the fabrication of biomimetic skin *in vitro*, one of the primary concerns is the reproduction of the functions of this intricate organ.

Table 2. Ideal properties *in vitro* 3D skin model development [101, 112, 114].

Ideal *in vitro* 3D skin model properties	Functions
Structure similarity	Mimic to human native skin.
Mechanical	Ability to withstand resistance to natural loading conditions such as tissue remodelling, deformation resistance, viscoelasticity comparable to natural skin, and the range of elastic modulus is from 10 to 850 kPa.
Biocompatibility	Encourage the attachment of cells, their proliferation and differentiation, and the regulation of both intercellular and intracellular communication.
Physicochemical	High crosslinking stability, intermolecular strength, resistance to degradation, porosity for nutrition and waste movement, and physiological pH and polymerizing temperature.
Moisture content	Minimum *in vitro* culture contraction and long-term hydration, and influence biodegradability of the model.

Full-thickness skin equivalent (FTSEs) closely resemble natural skin due to its bi-layered construction, including both the dermis and epidermis. Generally, the dermal part of FTSEs can be constructed without scaffolds, using self-produced extracellular matrix (ECM) from spheroids or cell sheets, or using native scaffolds such as de-epidermized dermises (DEDs), or manufactured scaffolds made from polymerized hydrogels [100]. However, it is imperative that the *in vitro* 3D skin model accurately represents the histological and anatomical properties of human skin, encompassing the epidermis, dermis and hypodermis. Moreover, reconstructing the dermis with polymerized hydrogels may enable efficient and reproducible FTSE generation compared to scaffold-free and DED skin models [101].

Apart from that, efforts have been made in developing *in vitro* 3D skin model for *in vitro* toxicity testing. The standard procedure for constructing *in vitro* 3D skin model involves creating a dermal scaffold that is loaded with fibroblasts and then cultivated for a period of several days to a week. Subsequently, keratinocytes migrate and settle on the dermal scaffold [102]. Similar to reconstructed human epidermis (RHEs), *in vitro* 3D skin model undergoes a brief submerged culture before air-lift and keratinocyte differentiation. Following that, a structure resembling the epidermis is generated by an ongoing process for almost 2 weeks. This structure has been proven to consistently replicate the physical characteristics (including well-organized layers such as basal, spinous, granular and corneal layers), chemical composition, and functional features of the human epidermis [103].

Mechanical properties

The mechanical characteristics of the skin influence dermal cell activity and affect tissue function. In the field of developing *in vitro* 3D skin model, many biomaterials have been utilized. Gelatinous skin models can interact with water, allowing for reversible gel production, allowing for manipulation of physical, mechanical and chemical properties like elastic modulus, hardness, optical, or surface qualities [104]. Among them, collagen type I has gained significant popularity due to its abundance in the dermal extracellular matrix (ECM) and its ease of extraction from natural sources [105]. However, collagen type I gels have poor mechanical characteristics and are easily influenced by fibroblast contraction. In recent years, the integration of two or more natural polymers has been utilized in addition to chemical cross-linking and non-enzymatic glycation as methods to enhance the stability of scaffold matrices [106]. Previous study has constructed *in vitro* 3D skin model using collagen type-I utilizing different crosslinking agents: a natural one called genipin (GNP) and a synthetic one called carbodiimide (EDC) [13]. After the crosslinking process, human skin cells were cultured on the bioscaffold under two different conditions: submerged (SM) and ALI. However, the findings indicate that EDC exhibits a higher level of toxicity towards the cell compared to GNP.

Besides, crosslinking silk and collagen has been said to work very effectively. This is because collagen has regions that hold cells together, and silk has better flexibility [107].

In addition, polydimethyl siloxane (PDMS) and polyvinyl alcohol (PVA) hydrogel cross-linked with glutaraldehyde (GA) has emerged as a potential of *in vitro* 3D skin models [108]. The indentation results obtained were consistent with those reported in the literature for the target epidermis, which exhibited an elastic modulus ranging from 0.1 to 1.5 MPa and was water-dependent. Synthetic polymers have been explored as dermal scaffolds due to their adjustable features, including porosity and elasticity. Cells adhere poorly to synthetic polymers, hence they are often used in conjunction with natural polymers for dependable *in vitro* skin function.

Moreover, a previous study has employed gelatine-hydrogel to sustain 3D skin [109]. This study found that mimicking human body circumstances and qualities is challenging, and gelatine-hydrogels' poor mechanical properties and rapid breakdown make them unsuitable for 3D *in vitro* cell growth. Nevertheless, this study suggested that by increasing the concentration of hydrogels and crosslinker (genipin) may resolve this issue. Additionally, peptide-based hydrogels represent a promising strategy for tissue engineering 3D model [110]. The mechanical stability of both peptide nanogels were enhanced due to the presence of a nanofiber network.

Moisture content

Besides providing a physiologically realistic environment for cells, hydrogel scaffolds' hydration and swelling qualities impact mechanical properties during biological gradients or stress [111]. The swelling ratio determine the capacity of the skin model to absorb fluid when it comes in contact with a liquid. To imitate the hydration condition of human skin, the model should retain an acceptable moisture content. As a result, research into hydrogel degradation rates and their potential effects on cellular physiology and function as a result of swelling behaviour is gaining interest. Porosity of the hydrogel, crosslinking density, and environmental parameters such as pH and temperature are frequently the factors that determine the swelling behaviour of the material [112]. Hydrogel swelling and water absorption capacity indicate hydrophilicity, whereas relative cross-linking density reflects network stiffness [113]. Hydrogels with optimal swelling are soft, while stiffer gels have lesser swelling. The swelling and water absorption of a gel can affect bioink system cellular activity since optimal swelling is necessary for nutrient transport.

Biocompatibility

Biocompatibility refers to a material's capacity to sustain live cells without altering their physiology or viability [114]. Briefly, an ideal *in vitro* skin model should promote cell adhesion and proliferation while being biocompatible and functional. In order to mimic native skin tissue, skin constructs should mimic cell-cell interactions with their pericellular ECM, regulating communication through bound growth factors, enzymes and bioactive species such as exosomes. The biocompatibility of the hydrogel and its mechanical features are closely linked in skin tissue engineering. This is because cells need to be able to adhere to the hydrogel for the tissue to grow.

The use of biocompatible natural or synthetic scaffolds for extracellular matrix (ECM) and dermal equivalents has been facilitated by recent advancements in tissue engineering [115]. Natural-based biopolymers have advantages over synthetic biopolymers due to their remarkable similarity to the composition of the human ECM. They mimic the natural environment of cells to promote cell adhesion, proliferation, migration and differentiation. For example, poly(L-lactic acid) (PLLA) and collagen type I skin equivalent showed a superior cell adhesion activity on the scaffold [116]. The usage of natural-based bioinks, such as alginate, gelatine and collagen, exhibit desirable biocompatibility, accelerated biodegradation, lack of toxicity, and optimal mechanical stability. A previous study also demonstrated that human skin cells have excellent biocompatibility with gelatine-polyvinyl alcohol 3D *in vitro* skin construct [45]. In addition, a three-dimensional skin model that was composed of fibrinogen and alginate exhibited a homogenous blend with a shear-thinning characteristic, outstanding printability, appropriate mechanical stiffness, porosity, biodegradability and water absorption, and it was shown to be *in vitro* biocompatible [117]. After printing, a skin equivalent dyed by live/dead and histological inspection revealed a tissue-like structure with two layers of living and proliferating cells on days 7 and 14 of culture.

Sustainability of *in vitro* 3D skin model

The *in vitro* 3D skin models have been developed to fulfil the requirements of both fundamental and practical research. In order for tissue-engineered skin model to be successfully applied, it is necessary for the morphological and ultrastructural organization of the epidermis, dermis and dermal–epidermal junction (DEJ) to be as similar as feasible to the structure of the native skin [118]. However, sustaining the stability of *in vitro* 3D skin as a culture model is critical for reliable and reproducible experimental outcomes and this includes toxicology study.

3D skin culture

The bioink's physical stability and tissue-specific microenvironment enhanced epidermal organization, dermal ECM secretion, and barrier function. To maintain cell viability in a *in vitro* 3D skin model, proper selection of culture conditions, including cell seeding ratio, density, techniques and culture medium (with or without supplements and growth factors), is essential. In in-vitro skin culture, serum supplementation can stimulate or inhibit keratinocyte development, leading to the usage of low-serum or serum-free medium for *in vitro* skin culture [119–121]. By avoiding serum in the media, skin model culture can be significantly enhanced. The production of *in vitro* skin models requires a complicated mixture of nutrients and growth factors; yet the immunocompetent skin equivalents that have been reported up until this point have utilized medium that is appropriate for the development of skin.

Previous research has investigated the impact of culture medium on the maturation of *in vitro* skin, as well as the phenotype and functioning of macrophages [122]. The purpose of this investigation was to get a better understanding of the role that culture circumstances play in the development of a functional immunocompetent *in vitro* skin model in the future. Cultured of skin models demonstrated reduced viability, transformed cell shape, and decreased sensitivity to pro- and anti-inflammatory stimuli in 2D and 3D, affecting skin maturation. The study suggests that immunocompetent skin models require a particular culture medium to accurately detect immune responses, making it essential for developing physiologically realistic models. Moreover, the model also serves as a structural support for the organotypic culture [123]. Thus, it must allow facilitating the effective transfer of nutrients, metabolites and test chemicals. The pore size was specifically chosen to inhibit undesired cell migration. Enough nutrients, growth hormones and regulatory elements may be transported *via* this porous scaffold to support cell division, proliferation, and survival [124]. In addition to facilitating the ongoing provision of nutrients and elimination of metabolic waste, dynamic perfusion is believed to generate shear stresses that stimulate epidermal maturation and regulate its barrier function [125,126]. Thus, a new perfusion device is needed to study the physiology and pathology of full-thickness skin and its appendages. This device should facilitate assembly, preserve sterility, provide sufficient oxygen and nutrients, and expose cultured skin to mechanical stimuli [127].

Human skin equivalents (HSEs), which were first developed over 30 years ago, have seen improvements in cell culture methods, medium, and advanced cell biology tools such as genetic engineering and cell reprogramming [100]. However, grafting epidermal sheets enriched in in holoclone-forming keratinocytes showed long-term graft survival but slow dermal renewal, causing wound contraction and instability. In order to sustain the skin model in the cultivation state and to avoid physical harm, 3D skin model need to be counterparts with care. Reduce interruptions during media changes and other operations to avoid disrupting the model's structure and integrity. In order to sustain cells beyond the confines of an organism's body, it is critical to have the proper provisions and apparatus to create an environment conducive to cell division and survival. The necessary equipment for this process includes a biosafety hood to ensure the safety of cultures and the individuals handling them, a cell incubator set at a temperature of 37 °C and 5% CO_2 levels for proper storage, a refrigerator for culture media and supplies, as well as flasks or petri dishes for cell placement, among other essential equipment [128,129]. Moreover, sustainable culture practices also encompass ethical considerations related to cell sourcing, consent and animal welfare. Ensuring that cell sources are ethically obtained and that research practices adhere to ethical guidelines promotes sustainability and social responsibility in research.

Besides, in a long-term culture model, the selection of biomaterials that facilitate cell adhesion, proliferation and differentiation is very critical to ensure that the materials utilized are stable over time and biocompatible. Biomaterials that are biocompatible and do not trigger an immune response or have cytotoxic effects should be used to develop skin model [130]. The use of materials that preserve the viability and health of cells over an extended period is necessary to ensure stability and durable over time. Generally, materials that have low mechanical strength tend to degrade faster. For example, gelatine is capable of dissolving in water, hence it must undergo crosslinking to improve its stability for use in tissue engineering [2]. Nevertheless, the primary issue associated with collagen biomaterials is their poor mechanical qualities and rapid breakdown rate [124,131]. Thus, crosslinking methods based on chemicals, UV light and enzymes or combining collagen with other synthetic polymers are used to make collagen structures stronger and more stable [132].

Primary human keratinocytes and fibroblasts are often used in skin models. The ideal cell seeding density for generating a 3D skin equivalent is depending upon several aspects, such as the particular cell type, intended usage, and the required thickness and complexity of the skin model. There have been previous publications that have demonstrated that the cell density of keratinocytes and fibroblasts ranged from 0.3 million to 5 million cells/mL [31,133]. Additionally, previous research has indicated that the extrusion-based bioprinting approach necessitates a high cell density in order to perform the printing process. A study has demonstrated a comparison of *in vitro* 3D skin model performance between 1.5×10^6 with and 3.0×10^6 cells/mL utilizing gelatine-polyvinyl alcohol bioinks [45]. The results demonstrated that cell densities of 3.0×10^6 cells/mL exhibited superior performance in terms of both cell survival and protein expression up to 14 days prolonged culture. This demonstrates that increasing cell seeding densities enhance cell-cell interactions in the bioinks. The long-term culture model sustainability of the *in vitro* 3D skin model is demonstrated in Table 3.

Advancing toxicity testing: the advantages of in vitro 3D skin models

The sustainability of *in vitro* 3D skin models in toxicity testing is an important consideration to develop more environmentally friendly and ethical approaches to assessing chemical

Table 3. Sustainability of the *in vitro* 3D skin model as a long-term culture model [45, 119–122, 130].

Sustainability of *in vitro* 3D skin model	Descriptions
Media selection	Select a culture medium that caters to the unique requirements of the 3D skin model.
Nutrient and oxygen supply	Periodically refresh the medium with new nutrients and growth elements to create the best possible environment for cell survival and activity.
Handling technique	Careful while handling 3D skin counterparts to avoid harm. Reduce model structure and integrity interruptions during media changes and other activities.
Proper selection of biomaterials	To facilitate cell adhesion, proliferation and differentiation and to sustain long-term cell culture model.
Optimal cell seeding density	To promote cell-cell interaction.

safety. The employment of *in vitro* 3D skin models allows researchers to reduce reliance on animal testing for assessing chemical toxicity. This reduction aligns with ethical considerations and contributes to the reduction of animal suffering. On the other hand, the *in vitro* 3D skin models require fewer resources compared to traditional animal testing methods. They typically consume fewer materials and reagents, reducing waste generation and minimizing the environmental impact associated with laboratory experiments.

In addition to reducing animal testing, *in vitro* 3D skin models offer an alternative to human testing for evaluating chemical toxicity. This approach minimizes the need for human volunteers in clinical trials, thereby addressing ethical concerns and avoiding potential risks to human health. Moreover, the *in vitro* 3D skin models can be adapted for high-throughput screening of large chemical libraries, this enables the efficient evaluation of toxicity profiles for numerous compounds [6]. This scalability improves the efficiency of toxicity testing while reducing the overall number of experiments required.

In vitro skin models that are 3D in nature are superior to typical 2D cell cultures in terms of their ability to replicate the physiological structure and complexity of human skin. In many cases, they consist of a number of different cell types and layers, which allows them to provide a more accurate visualization of the structure of the skin. Furthermore, a significant limitation of these skin equivalents remains their absence of a vascular system, which hinders the delivery of nutrients, oxygen, waste elimination and nutrient concentration gradient [134].

The *in vitro* 3D skin models provide notable benefits in terms of physiological accuracy and predictive capability, but it is necessary to tackle issues associated with uniformity, cost and technical proficiency. Comparatively, 3D models have demonstrated higher precision in predicting *in vivo* responses than 2D models. Therefore, ongoing efforts have been undertaken to achieve this goal, resulting in notable advancements in the creation of *in vitro* 3D skin models for both the epidermis and dermis. These models have been developed employing diverse biomaterials, including collagen type I, extracellular matrix (ECM), fibrin and de-epidermized dermal matrix. Development of the *in vitro* 3D skin models is comparatively rapid, reasonably priced, employs high-throughput techniques and matches well with *in vivo* clinical studies [135].

On the aspect of human skin physiology and responses, the *in vitro* 3D skin models enable researchers to optimize chemical testing protocols, leading to reduced chemical usage. This reduction contributes to lower costs and decreases environmental impact associated with chemical production and disposal. Additionally, the *in vitro* 3D skin models offer improved predictive accuracy compared to traditional 2D cell cultures, providing more reliable assessments of chemical toxicity. This enhanced accuracy reduces the need for repeat experiments and minimizes unnecessary exposure to chemicals, further promoting sustainability in toxicity testing [136].

The *in vitro* 3D skin models are also increasingly recognized and accepted by regulatory agencies as valid alternatives for assessing chemical toxicity. This recognition encourages the adoption of sustainable testing methods and facilitates the implementation of alternative testing strategies in regulatory frameworks worldwide. Most importantly, the sustainability of *in vitro* 3D skin models in toxicity testing is evident through their contributions to reducing animal testing, resource efficiency, high-throughput screening capabilities, reduced chemical usage, predictive accuracy and regulatory acceptance. Embracing these sustainable approaches not only benefits the environment and animal welfare but also promotes the development of safer products and chemicals for human health.

Machine learning using in vitro 3D skin model for toxicity testing

Machine learning (ML) is increasingly being utilized in conjunction with *in vitro* 3D skin models for skin toxicity testing, providing a more ethical and effective approach to assessing the safety of chemicals, cosmetics and pharmaceuticals. ML enhances the predictive capabilities of these 3D skin models by analyzing large datasets to identify patterns and correlations that may not be evident through traditional testing methods. For instance, ML models have been developed to predict skin sensitization potential, which is crucial for evaluating the safety of new compounds. These models utilize molecular descriptors and various classification techniques, such as random forests and support vector machines, to provide reliable predictions of skin reactions [137]. The application of machine learning (ML) in skin toxicity testing not only enhances the accuracy and efficiency of assessments but also aligns with key Sustainable Development Goals (SDGs) by promoting health, innovation and ethical standards in chemical safety.

Identifying good quality cells

Deep learning is utilized in medical imaging and drug toxicology, utilizing structural alterations in cells or tissues induced by toxicity. ML algorithms can enhance the reliability of toxicity assessments by analyzing cell images, identifying high-quality cells and training models on features like cell morphology and viability. Previously, this approach is used *in vitro* to assess toxicity by examining changes in fluorescently labelled cell nuclei and detecting small structural alterations in cardiomyocytes and hepatocytes [138]. The study demonstrates that the computed epidermal thickness from the EpiDerm model can accurately reflect potential clinical skin toxicity, demonstrating its automation, scalability and reproducibility [139].

Optimizing the fabrication process

ML techniques enable real-time monitoring of skin model responses to toxic substances, providing insights into cellular changes like gene expression or metabolic activity, enabling immediate adjustments in experimental conditions. The process of optimizing the fabrication of *in vitro* skin models using machine learning involves several stages, including data collection, preprocessing, model training and deployment. Furthermore, ML may improve 3D bioprinting by recommending more effective bioinks, 3D bioprinters and software, as well as learning complicated instructions and optimizing manufacturing processes through parameter optimization and condition prediction [140]. A study has developed a system using convolutional neural networks to detect anomalies in hydrogel-based bioink, aiming to

improve tissue composition through real-time autonomous correction in 3D bioprinting, thereby enhancing the quality of the final product [141]. The research aims to improve tissue composition in 3D bioprinting by processing images into small patches with grid, gyroid, rectilinear and honeycomb shapes. ML can enhance bioprinting by real-time monitoring, detecting approximate dimensions and curved layers, and correcting incorrect bioink positioning and microstructure errors. When it comes to extracting representative features about the edges and lines on the objects, the convolutional neural network has a major benefit compared to position-invariant features.

Moreover, biofabrication involves scaffolds as a microenvironment for cell growth and differentiation in tissue structures. These complex structures involve active interaction between cells and biomaterials, cell adhesion, and nutrient transport for cell growth, making them essential for tissue engineering. Thus, ML can accurately predict the success rate for building scaffolds, enabling the achievement of high-quality outcomes [142]. A prior work used training data on printing parameters and bioink concentrations to construct a probabilistic model that suggested an optimal printer configuration using a filament scoring system [143].

Real-time monitoring

ML techniques enable real-time monitoring of skin model responses to toxic substances, providing insights into cellular changes like gene expression or metabolic activity, enabling immediate adjustments in experimental conditions.

Predictive modelling

ML models are utilized to predict skin toxicity from chemical compounds, significantly reducing the reliance on traditional animal testing methods. For instance, studies have shown that ML algorithms can accurately assess skin irritation and corrosion potential using datasets derived from *in vivo* classifications, enhancing the efficiency and ethical considerations of toxicity testing. To create datasets for machine learning models in predictive toxicology, popular databases to employ are PubChem, ToxCast and ChEMBL [144]. These databases include information on several chemical groupings. For instance, ToxCast mostly focuses on industrial chemicals, whereas ChEMBL contains data for molecules that resemble drugs [145,146]. Chemical structures in predictive toxicology are often represented by attributes that ML algorithms can process. Predictive toxicology employs machine learning approaches to model chemical structures using molecular descriptors, fingerprints, or both, which are often derived *via* cheminformatics toolkits such as atom count, logP and solubility [147].

Ethical testing

This approach aligns with the 3Rs principle (Replace, Reduce, Refine) by minimizing the need for animal testing and providing a more humane alternative for toxicity assessments. By reducing the need for animal testing, ML applications in skin toxicity testing address ethical concerns while also lowering costs associated with traditional testing methods. This supports responsible consumption and production patterns, contributing to the SDGs focused on sustainable practices [148].

Regulatory compliance

As regulatory frameworks increasingly favour non-animal testing methods, the integration of machine learning with 3D skin models positions researchers to meet these evolving standards effectively. The development of ML models that meet regulatory standards for toxicity testing can facilitate the approval process for new chemicals and drugs. This ensures that safety assessments are both efficient and compliant with health regulations, further promoting public safety and environmental protection. Figure 5 below demonstrated the illustration image of the usage of ML in skin toxicity testing utilizing *in vitro* 3D skin model.

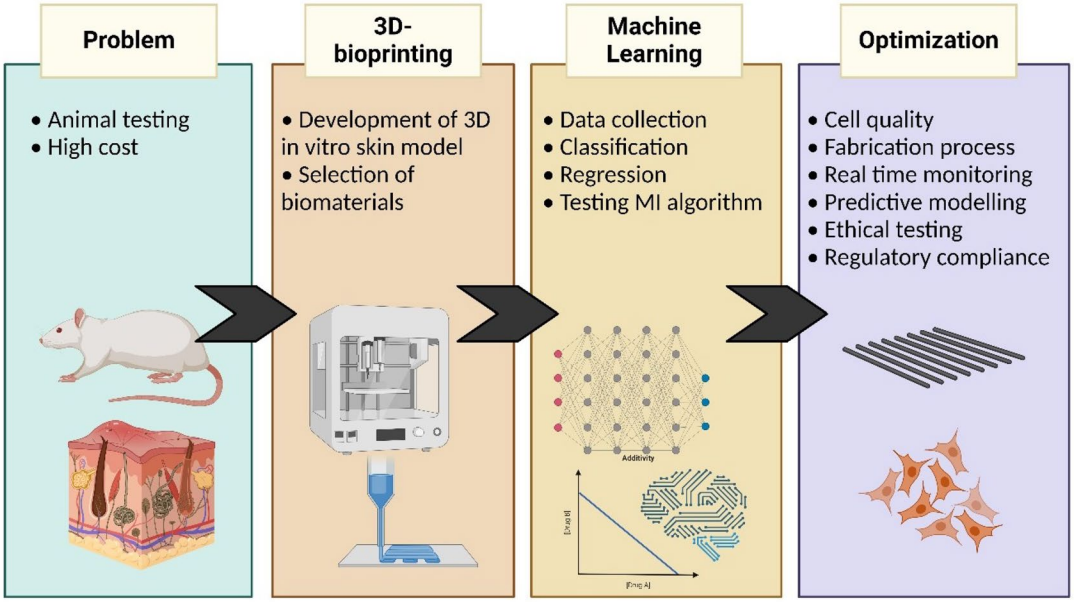

Figure 5. A Schematic illustration of utilizing ML to develop *in vitro* 3D skin model for toxicity testing. This ML approach able to optimize and identify the cell quality, fabrication process, real time monitoring, predictive modelling, ethical testing and regulatory compliance. Image created using Biorender.com.

In vitro vs in vivo skin toxicity

The assessment of skin toxicity involves the use of two distinct approaches, including *in vitro* and *in vivo* study. Preclinical testing within the pharmaceutical industry encompasses two distinct phases: *in vivo* investigations on pertinent animal models during the late discovery phase and *in vitro* evaluations on suitable cell line models during the early stages of development. Toxicity testing typically involves observing adverse health outcomes in animal models at high doses to determine a no-observed-effect level (NOEL) or no-observed-adverse-effect level (NOAEL). If the NOEL/NOAEL is not clear, the lowest observed-effect level (LOAEL) is used for extrapolation to expected effects [149]. The Integrated method on Testing and Assessment (IATA) for skin corrosion and irritation by Organization for Economic Cooperation and Development (OECD) aims to decrease animal testing and standardize chemical safety assessment. It provides a tiered method for evaluating directly applied (topical) substances [150]. The IATA for skin corrosion and irritation has three components: an evaluation of existing data (including human and animal studies, physicochemical parameters, quantitative structure activities), a weight of evidence (WoE) analysis, and a plan for additional testing. The primary objective of the tiered approach to skin corrosion and irritation evaluation outlined in the International Air Transport Association (IATA) is to highlight the utilization of animal testing as a final option [4]. Several *in vitro* tests have been approved by regulatory bodies in the US and Europe. However, a combined approach to safety assessment for drugs that replaces all animal testing with *in vitro* tests is yet to be developed and approved [151].

In vitro

Currently, reconstructed epidermis models have exhibited enhanced permeability in comparison to excised human skin and porcine skin, which are frequently employed as surrogate models for dermal absorption in the field of pharmaceutical research. Several *in vitro* cytotoxicity tests have been developed to assess the acute toxicity of chemical substances. To reduce the need to employ animals in absorption, distribution, metabolism and excretion (ADME) determination, new *in vitro* alternative test models should be investigated. Animals are used in preclinical drug trials to find the median fatal doses (LD50). The therapeutic index, which measures the medicine's selectivity in achieving its desired effects, is calculated as the ratio of the LD50 to the median effective doses [149]. Whether the *in vitro* studies are based on immortalized (e.g. SV40 transformation) or cancer-derived cell lines, stem cells, reconstituted tissue cultures, or primary cells, the *in vivo* mechanisms of action of toxic compounds in humans must be suitably imitated *in vitro* systems. Cells or tissue may no longer display the necessary *in vivo*-like functionality for testing compounds, leading to false negative responses or false positive responses in healthy individuals, or may express mechanisms that are not active *in vivo*. One typical endpoint in *in vitro* toxicity assessment is the evaluation of cell viability. This entails evaluating the number of live cells following exposure to a chemical. Methods like MTT test, ATP assays, and others are often applied for this purpose.

The traditional two-dimensional (2D) cell culture approach, which has been used for a long time, has been demonstrated to be an effective technique for rapidly identifying hazardous chemicals. Nevertheless, the limitations of 2D culture are becoming more widely acknowledged. Due to very artificial geometric and mechanical limitations that are imposed on cells, two-dimensional cell cultures can only approximate the qualities of normal tissues [152]. Furthermore, this approximation is always restricted to single cell types and does not take into consideration the influence of other cells or the environment. The human skin equivalents (HSEs) and three-dimensional (3D) *in vitro* products were established with the purpose of conducting *in vitro* skin testing of pharmaceutical products and developing viable skin replacement alternatives for patients afflicted with diverse skin conditions, including burn victims [153]. Currently, HSEs are epidermal replacements made of keratinocytes in 3D cell culture models like Epiderm™, SkinEthic RHE™ and EpiSkin™, utilized for evaluating topical product irritation in pharmaceutical and cosmetic industries [154]. Due to their epidermal layer and keratinocyte origin, these skin equivalents are limited for evaluating products for immune system-related skin disorders, such as wound healing. Meanwhile the EpidermFT™ is known as full-thickness skin model, which combines both normal human keratinocytes and fibroblasts cultivated into a 3D model of several layers of the epidermis and dermis to replicate human skin more closely for toxicity testing. Nowadays, organizations and regulatory agencies are increasingly endorsing the development of *in vitro* toxicity testing procedures as replacements for traditional animal testing in regulatory frameworks. This aims to decrease the reliance on animal testing.

In vivo

In vivo skin toxicity testing uses living organisms to assess chemical effects on the skin. For a more comprehensive and complex assessment of chemicals' effects on the skin, *in vivo* testing is done on living creatures, commonly animals, rather than *in vitro* testing on isolated cells or tissues. *In vivo* studies identify the therapeutic index and examine toxicity, absorption, distribution, metabolism and excretion (ADME) qualities, which are critical for medication safety and possible utility [149]. Initially, *in vivo* investigations were conducted with the purpose of predicting acute systemic toxicity, often in rats. Presently, toxicological studies, particularly for regulatory testing, employ more advanced, focused and diverse methods that have well defined goals and experimental procedures. Despite the higher cost compared to other approaches, animals are still used in experiments due to numerous significant reasons [155]. Firstly, there is much information about their typical biochemical and physiological features. Second, *in vivo* toxicological endpoint data from relevant models for people and domestic/farm animals makes animal testing a helpful tool for predicting toxicity.

In vivo experiments for cutaneous toxicity are intended to investigate acute, sub chronic and chronic systemic toxic effects and a potential acute local irritation [155]. In cutaneous toxicity testing, animals are observed for skin responses, clinical findings and pathological findings, depending on the length and observation time of hazardous substance administration. The albino

rabbit was once the preferred animal model due to its high permeability and susceptibility to hazardous chemicals, which occasionally led to overprediction and little relation to human irritation [156]. Currently, albino rats and occasionally guinea pigs are preferred to evaluate local irritation. Traditionally, sensitization testing has been conducted on guinea pigs and mice. Nevertheless, investigations on the long-term toxicity of the skin frequently include models of rodents (such as albino rats and mice) as well as nonrodent animals. The physiological similarities between minipig skin and that of humans and pigs make minipigs a dependable nonrodent species.

Challenges in advancing in vitro 3D skin models for toxicity testing

While *in vitro* 3D skin models hold significant promise for toxicity testing, several challenges must be addressed to fully realize their potential. Additionally, future advancements and perspectives can further enhance their utility in this field. Establishing standardized protocols and quality control measures for *in vitro* 3D skin models presents a challenge, particularly in ensuring consistent reproducibility and reliability across various laboratories and studies. In addition, replicating the complexity of native skin tissue, which encompasses its multi-layered structure, intricate cell-cell interactions, and barrier function, remains a challenging approach in the *in vitro* 3D skin models. Interestingly, the challenge of *in vitro* 3D skin models lies in accurately replicating the intricate features of native skin tissue, such as its multi-layered structure, cell-cell interactions and barrier function.

The maintenance of the stability and functionality of *in vitro* 3D skin models over extended culture periods poses a significant challenge, particularly when conducting long-term toxicity studies or chronic exposure assessments. These models aim to mimic the complexity of native skin tissue, including its multi-layered structure, cell-cell interactions and barrier function. However, ensuring their viability and reliability over time requires careful optimization of culture conditions, media formulations and scaffold materials. Additionally, developing high-throughput screening platforms using *in vitro* 3D skin models necessitates the implementation of automation and miniaturization techniques to increase throughput while maintaining assays sensitivity and reliability. Furthermore, integrating immune cells into these models to replicate the skin's immune response to chemical exposures poses technical challenges but is crucial for accurately assessing immune-mediated toxic effects. In order to gain widespread acceptance and regulatory approval, extensive validation studies are necessary to demonstrate the predictive value and reliability of *in vitro* 3D skin models for toxicity testing. Collaboration among academia, industry and regulatory agencies is essential to address these challenges and establish standardized protocols for their use in safety assessment and regulatory decision-making [157].

The future landscape of in vitro 3D skin models in toxicity testing

The future of *in vitro* 3D skin models in toxicity testing holds immense promise, driven by ongoing advancements and evolving perspectives. Integration of *in vitro* 3D skin models with other organotypic models, such as liver, lung and kidney, into multi-organ systems, like "organ-on-a-chip" platforms, promises more comprehensive toxicity assessments and better prediction of systemic effects from chemical exposures [158]. Furthermore, advancements in personalized medicine and stem cell technologies open avenues for developing patient-specific *in vitro* 3D skin models, allowing for individualized toxicity testing and personalized risk assessment. In addition, incorporating microfluidic systems into these models enables dynamic culture conditions, perfusion-based culture and controlled exposure to chemicals, thereby enhancing physiological relevance and predictive value [6]. Moreover, leveraging advanced imaging techniques and high-content analysis platforms facilitates real-time monitoring of cellular responses and precise characterization of tissue morphology and function in *in vitro* 3D models. Researchers could complement experimental data with computational modelling approaches like quantitative structure-activity relationship (QSAR) and physiologically based pharmacokinetic (PBPK) modelling to enhance predictive toxicology assessments and reduces reliance on animal testing [159]. To date, by embracing these advancements and addressing associated challenges will expedite the usage of *in vitro* 3D skin models integration as reliable, cost-effective and ethical alternatives in skin toxicology study and regulatory applications.

Conclusions

The aim to establish an *in vitro* 3D skin model as a reliable and predictive pre-clinical tool for human skin safety assessment is greatly important and challenging. Studies showed that *in vitro* 3D skin model response data used as the *in vivo* basis evaluation of *in vitro* responses against chemicals and product formulations. Understanding the pathophysiological changes in the 3D skin model and the smart design of the model serve the sustainability in toxicology studies. Limitations to the use of *in vitro* 3D skin model in skin safety assessment have been identified in many studies. There are many drawbacks using *in vitro* 3D model under the OECD TG programme, such as lack of sufficient metabolic capacity, limited set of cell types and the requirement for the test materials to be soluble in aqueous buffers or cell culture media.

The challenges arise from developing alternative methods results to their improvement as well as to the search for new opportunities. Additionally, market demand or legislation greatly contribute to the development of alternative methods. The search takes place for the most efficient research system among primary or continuous cell lines but also 3D models such as equivalents of the human epidermis or skin, co-culture methods may also be promising. The increasing knowledge underlying mechanism in skin toxicology is the basis for the establishment of the alternative methods as we as Defined Approaches/Integrated Approaches to Testing and Assessment [160]. However, with the advancement of technologies in developing *in vitro* 3D skin model is expected to provide accurate placement of all various native skin cell types and play an important tool in toxicology in the context of risk assessment.

Author contributions

Syafira Masri: Conceptualization, data collection, methodology and writing – original draft; Mh Busra Fauzi: Conceptualization, validation and reviewing; Nor Fadilah Rajab: Conceptualization, interpretation of data and reviewing; Wing-Hin Lee: Conceptualization, design, validation of data and reviewing; Diana Atiqah Zainal Abidin: Data collection, analysis, reviewing and editing; Ee Ling Siew: Conceptualization, resources, design, funding acquisition, writing – original draft, editing and final approval of the version to be published. All authors have read and agreed to the published version of the manuscript.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

The authors thank the Ministry of Higher Education (MOHE) Malaysia for the financial support provided *via* the Fundamental Research Grant Scheme (FRGS/1/2021/STG01/UKM/02/1).

ORCID

Syafira Masri http://orcid.org/0000-0002-0282-0294
Mh Busra Fauzi http://orcid.org/0000-0001-6449-639X
Nor Fadilah Rajab http://orcid.org/0000-0002-2946-5528
Wing-Hin Lee http://orcid.org/0000-0003-0212-6523
Ee Ling Siew http://orcid.org/0000-0002-3420-5114

Data availability statement

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

References

[1] Pedrosa TdN, Catarino CM, Pennacchi PC, et al. Toxicity assessment. In 2021. doi: 10.1201/9781439805237-9.
[2] Sheikholeslam M, Wright MEE, Jeschke MG, et al. Biomaterials for skin substitutes. Adv Healthc Mater. 2019;176(5):139–148. doi: 10.1002/adhm.201700897.Biomaterials.
[3] Masri S, Fauzi MB. Current insight of printability quality improvement strategies in natural-based bioinks for skin regeneration and wound healing. J Polymers. 2021;13(7):1011. doi: 10.3390/polym13071011.
[4] Hardwick RN, Betts CJ, Whritenour J, et al. Drug-induced skin toxicity: gaps in preclinical testing cascade as opportunities for complex: in vitro models and assays. Lab Chip. 2020;20(2):199–214. doi: 10.1039/c9lc00519f.
[5] Hartung T. Toxicology for the twenty-first century. Bryol Twenty First Century. 2009;460:1–382. doi: 10.1201/9781315138626.
[6] Ng WL, Yeong WY. The future of skin toxicology testing—Three-dimensional bioprinting meets microfluidics. Int J Bioprint. 2019;5(2.1):237. doi: 10.18063/ijb.v5i2.1.237.
[7] Liguori GR, Jeronimus BF, De Aquinas Liguori TT, et al. Ethical issues in the use of animal models for tissue engineering: reflections on legal aspects, moral theory, three Rs strategies, and harm-benefit analysis. Tissue Eng Part C Methods. 2017;23(12):850–862. doi: 10.1089/ten.tec.2017.0189.
[8] marketsandmarkets [Internet]. In vitro toxicology testing market. 2017. Available from: https://www.marketsandmarkets.com/PressReleases/in-vitro-toxicology-testing.asp
[9] Bojar RA. Studying the human skin microbiome using 3D in vitro skin Models. Appl In Vitro Toxicol. 2015;1(2):165–171. doi: 10.1089/aivt.2015.0002.
[10] Netzlaff F, Lehr CM, Wertz PW, et al. The human epidermis models EpiSkin®, SkinEthic® and EpiDerm®: an evaluation of morphology and their suitability for testing phototoxicity, irritancy, corrosivity, and substance transport. Eur J Pharm Biopharm. 2005;60(2):167–178. doi: 10.1016/j.ejpb.2005.03.004.
[11] Roguet R, Cohen C, Leclaire J, et al. Use of a standardized reconstructed epidermis kit to assess in vitro the tolerance and the efficacy of cosmetics. Int J Cosmet Sci. 2000;22(6):409–419. doi: 10.1046/j.1467-2494.2000.00016.x.
[12] Abd E, Yousef SA, Pastore MN, et al. Skin models for the testing of transdermal drugs. Clin Pharmacol. 2016;8:163–176. doi: 10.2147/CPAA.S64788.
[13] Fauzi MB, Rashidbenam Z, Bin Saim A, et al. Preliminary study of in vitro three-dimensional skin model using an ovine collagen type i sponge seeded with co-culture skin cells: submerged versus air-liquid interface conditions. Polymers (Basel). 2020;12(12):2784. doi: 10.3390/polym12122784.
[14] Jensen C, Teng Y. Is it time to start transitioning from 2D to 3D cell culture? Front Mol Biosci. 2020;7:33. doi: 10.3389/fmolb.2020.00033.
[15] Chen L, Wu M, Jiang S, et al. Skin toxicity assessment of silver nanoparticles in a 3D epidermal model compared to 2D keratinocytes. Int J Nanomed. 2019;14:9707–9719. doi: 10.2147/IJN.S225451.
[16] Nie J, Gao Q, Fu J, et al. Grafting of 3D bioprinting to in vitro drug screening: a review. Adv Healthc Mater. 2020;9(7):e1901773. doi: 10.1002/adhm.201901773.
[17] Kapałczyńska M, Kolenda T, Przybyła W, et al. 2D and 3D cell cultures – a comparison of different. Arch Med Sci. 2016;14(4):910–919. doi: 10.5114/aoms.2016.63743.
[18] Sutterby E, Thurgood P, Baratchi S, et al. Evaluation of in vitro human skin models for studying effects of external stressors and stimuli and developing treatment modalities. View. 2022;3(2):1–19. doi: 10.1002/VIW.20210012.
[19] Poumay Y, Dupont F, Marcoux S, et al. A simple reconstructed human epidermis: preparation of the culture model and utilization in in vitro studies. Arch Dermatol Res. 2004;296(5):203–211. doi: 10.1007/s00403-004-0507-y.
[20] Dey M, Ozbolat IT. 3D bioprinting of cells, tissues and organs. Sci Rep. 2020;10(1):14023. doi: 10.1038/s41598-020-70086-y.
[21] Choi J, Lee EJ, Jang WB, et al. Development of biocompatible 3D-printed artificial blood vessels through multidimensional approaches. J Funct Biomater. 2023;14(10):497. doi: 10.3390/jfb14100497.
[22] Jeong Y-M, Bang C, Park M, et al. 3D-printed collagen scaffolds promote maintenance of cryopreserved patients-derived melanoma explants. Cells. 2021;10(3):589. doi: 10.3390/cells10030589.
[23] Saydé T, El Hamoui O, Alies B, et al. Biomaterials for three-dimensional cell culture: from applications in oncology to nanotechnology. Nanomaterials. 2021;11(2):481. doi: 10.3390/nano11020481.
[24] Ng WL, Qi JTZ, Yeong WY, et al. Proof-of-concept: 3D bioprinting of pigmented human skin constructs. Biofabrication. 2018;10(2):025005. doi: 10.1088/1758-5090/aa9e1e.
[25] Derr K, Zou J, Luo K, et al. Fully three-dimensional bioprinted skin equivalent constructs with validated morphology and barrier function. Tissue Eng Part C Methods. 2019;25(6):334–343. doi: 10.1089/ten.tec.2018.0318.
[26] Wei Z, Liu X, Ooka M, et al. Two-dimensional cellular and three-dimensional bio-printed skin models to screen topical-use compounds for irritation potential. Front Bioeng Biotechnol. 2020;8:109. doi: 10.3389/fbioe.2020.00109.
[27] Gu Z, Fu J, Lin H, et al. Development of 3D bioprinting: from printing methods to biomedical applications. Asian J Pharm Sci. 2020;15(5):529–557. doi: 10.1016/j.ajps.2019.11.003.
[28] Miri AK, Mirzaee I, Hassan S, et al. Effective bioprinting resolution in tissue model fabrication. Lab Chip. 2019;19(11):2019–2037. doi: 10.1039/c8lc01037d.

[29] Ng WL, Shkolnikov V. Optimizing cell deposition for inkjet-based bioprinting. IJB. 2024;10(2):2135. doi: 10.36922/ijb.2135.
[30] Saunders RE, Gough JE, Ã BD. Delivery of human fibroblast cells by piezoelectric drop-on-demand inkjet printing. Biomaterials. 2008;29(2):193–203. doi: 10.1016/j.biomaterials.2007.09.032.
[31] Lee V, Singh G, Trasatti JP, et al. Design and fabrication of human skin by three-dimensional bioprinting. Tissue Eng Part C Methods. 2014;20(6):473–484. doi: 10.1089/ten.tec.2013.0335.
[32] Xu C, Zhang M, Huang Y, et al. Study of droplet formation process during drop-on-demand inkjetting of living cell-laden bioink. Langmuir. 2014;30(30):9130–9138.
[33] Nooranidoost M, Izbassarov D, Tasoglu S, et al. A computational study of droplet-based bioprinting : effects of viscoelasticity. Phys Fluids 2019;31(8):081901. doi: 10.1063/1.5108824.
[34] Tasoglu S, Kaynak G, Szeri AJ, et al. Impact of a compound droplet on a flat surface : a model for single cell epitaxy. Phys Fluids 2010;22(8):082103. doi: 10.1063/1.3475527.
[35] Ng WL, Huang X, Shkolnikov V, et al. Controlling droplet impact velocity and droplet volume: key factors to achieving high cell viability in sub-nanoliter droplet-based bioprinting. Int J Bioprint. 2022;8(1):424. doi: 10.18063/ijb.v8i1.424.
[36] Mandrycky C, Wang Z, Kim K, et al. 3D bioprinting for engineering complex tissues. Biotechnol Adv. 2016;34(4):422–434. doi: 10.1016/j.biotechadv.2015.12.011.
[37] Bagatin JdT, Camarena DEM, Osaki LH, et al. Bioprinted and manual human epidermis production: A compared performance for skin irritation tests. Bioprinting. 2023;29:e00251. doi: 10.1016/j.bprint.2022.e00251.
[38] Malekpour A, Chen X. Printability and cell viability in extrusion-based bioprinting from experimental, computational, and machine learning views. J Funct Biomater. 2022;13(2):40.
[39] England S, Rajaram A, Schreyer DJ, et al. Bioprinting bioprinted fibrin-factor XIII-hyaluronate hydrogel scaffolds with encapsulated Schwann cells and their in vitro characterization for use in nerve regeneration. Bioprinting. 2017;5:1–9. doi: 10.1016/j.bprint.2016.12.001.
[40] Nair K, Gandhi M, Khalil S, et al. Characterization of cell viability during bioprinting processes. Biotechnol J. 2009;4(8):1168–1177. doi: 10.1002/biot.200900004.
[41] Ouyang L, Yao R, Mao S, et al. Three-dimensional bioprinting of embryonic stem cells directs highly uniform embryoid body formation. Biofabrication. 2015;7(4):044101. doi: 10.1088/1758-5090/7/4/044101.
[42] Yang Y, Xu R, Wang C, et al. Recombinant human collagen-based bioinks for the 3D bioprinting of full-thickness human skin equivalent. Int J Bioprint. 2022;8(4):611. doi: 10.18063/ijb.v8i4.611.
[43] Jin R, Cui Y, Chen H, et al. Three-dimensional bioprinting of a full-thickness functional skin model using acellular dermal matrix and gelatine methacrylamide bioink. Acta Biomater. 2023;131(127):248–261. doi: 10.1016/j.actbio.2021.07.012.
[44] Qui´lez C, Gonzalo de Aranda I, Marta G, et al. 3D bioprinting principles and protocols. In: Ctook JM, editor. Methods in molecular biology. Springer; 2020. p. 221. doi: 10.1007/978-1-0716-0520-2_12.
[45] Masri S, Fauzi FAM, Hasnizam SB, et al. Engineered-skin of single dermal layer containing printed hybrid gelatin-polyvinyl alcohol bioink via 3D-bioprinting: in vitro assessment under submerged vs. air-lifting models. Pharmaceuticals. 2022;15(11):1328. doi: 10.3390/ph15111328.
[46] Schmidt FF, Nowakowski S, Kluger PJ. Improvement of a three-layered in vitro skin model for topical application of irritating substances. Front Bioeng Biotechnol. 2020;8:388. doi: 10.3389/fbioe.2020.00388.
[47] Kreimendahl F, Ko M. Three-dimensional printing and angiogenesis . Tissue Eng - Part C. 2017;23(10):604–615. doi: 10.1089/ten.tec.2017.0234.
[48] Camacho P, Busari H, Seims KB, et al. Materials as bioinks and bioink design. In 3D bioprinting in medicine. Springer; 2019. p. 67–99. doi: 10.1007/978-3-030-23906-0.
[49] Kong JH, Smith MK, Mooney DJ. Designing alginate hydrogels to maintain viability of immobilized cells. Biomaterials. 2003;24(22):4023–4029. doi: 10.1016/S0142-9612(03)00295-3.
[50] Chung JHY, Yue Z, Kapsa R, et al. Bio-ink properties and printability for extrusion printing living cells. Biomater Sci. 2013;1(7):763–773. doi: 10.1039/c3bm00012e.
[51] Long W, Min J, Wen Y, et al. Vat polymerization-based bioprinting - process, materials, applications and regulatory challenges. Biofabrication. 2020;12:022001. doi: 10.1088/1758.
[52] Xu HQ, Liu JC, Zhang ZY, et al. A review on cell damage, viability, and functionality during 3D bioprinting. Mil Med Res. 2022;9(1):70. doi: 10.1186/s40779-022-00429-5.
[53] Cros JM. HPV: The global burden. Nature. 2012;488:S2–S3.
[54] Xiang Y, Miller K, Guan J, et al. 3D bioprinting of complex tissues in vitro: state-of-the-art and future perspectives. Arch Toxicol. 2022;96(3):691–710. doi: 10.1007/s00204-021-03212-y.
[55] Koch L, Deiwick A, Schlie S, et al. Skin tissue generation by laser cell printing. Biotechnol Bioeng. 2012;109(7):1855–1863. doi: 10.1002/bit.24455.
[56] Zidarič T, Milojević M, Gradišnik L, et al. Polysaccharide-based bioink formulation for 3D bioprinting of an in vitro model of the human dermis. Nanomaterials. 2020;10(4):733. doi: 10.3390/nano10040733.
[57] Liu J, Zhou Z, Zhang M, et al. Simple and robust 3D bioprinting of full-thickness human skin tissue. Bioengineered. 2022;13(4):10087–10097. doi: 10.1080/21655979.2022.2063651.
[58] Rousi E, Malheiro A, Harichandan A, et al. An innervated skin 3D in vitro model for dermatological research. In Vitro Models. 2022;2(3-4):113–121. doi: 10.1007/s44164-022-00021-0.
[59] Hoeffel G, Debroas G, Roger A, et al. Sensory neuron-derived TAFA4 promotes macrophage tissue repair functions. Nature. 2021;594(7861):94–99. doi: 10.1038/s41586-021-03563-7.
[60] Moon S, Kim DH, Shin JU. In vitro models mimicking immune response in the skin. Yonsei Med J. 2021;62(11):969–980. doi: 10.3349/ymj.2021.62.11.969.
[61] Ma X, Wang F, Wang B. Application of an in vitro reconstructed human skin on cosmetics in skin irritation tests. J Cosmet Dermatol. 2021;20(6):1933–1941. doi: 10.1111/jocd.13789.
[62] Portugal-Cohen M, Cohen D, Kohen R, et al. Exploitation of alternative skin models from academia to industry: proposed functional categories to answer needs and regulation demands. Front Physiol. 2023;14:1215266. doi: 10.3389/fphys.2023.1215266.
[63] OECD. Test No. 431: in vitro skin corrosion: reconstructed human epidermis (RHE) test method. Paris: OECD Publishing; 2015. doi: 10.1787/9789264242753-en.
[64] Thélu A, Catoire S, Kerdine-Römer S. Immune-competent in vitro co-culture models as an approach for skin sensitisation assessment. Toxicol In Vitro. 2020;62:104691. doi: 10.1016/j.tiv.2019.104691.
[65] Lee H, Cheong KA, Kim JY, et al. Il-1 receptor antagonist reduced chemical-induced keratinocyte apoptosis through antagonism to il-1α/il-1β. Biomol Ther (Seoul). 2018;26(4):417–423. doi: 10.4062/biomolther.2017.167.
[66] Jusoh N, Ko J, Jeon NL. Microfluidics-based skin irritation test using in vitro 3D angiogenesis platform. APL Bioeng. 2019;3(3):036101. doi: 10.1063/1.5093975.
[67] Khan Y, Sadia H, Ali Shah SZ, et al. Nanoparticles, and their applications in various fields of nanotechnology : a review. Catalysts. 2022;12(11):1386. doi: 10.3390/catal12111386.
[68] Kim JS, Song KS, Sung JH, et al. Genotoxicity, acute oral and dermal toxicity, eye and dermal irritation and corrosion and skin sensitisation evaluation of silver nanoparticles. Nanotoxicology. 2013;7(5):953–960. doi: 10.3109/17435390.2012.676099.
[69] Paladini F, Sannino A, Pollini M. In vivo testing of silver treated fibers for the evaluation of skin irritation effect and hypoallergenicity. J Biomed Mater Res B Appl Biomater. 2014;102(5):1031–1037. doi: 10.1002/jbm.b.33085.
[70] Park Y-H, Kim JN, Jeong SH, et al. Assessment of dermal toxicity of nanosilica using cultured keratinocytes, a human skin equivalent model and an in vivo model. Toxicology. 2010;267(1–3):178–181. doi: 10.1016/j.tox.2009.10.011.
[71] Samberg ME, Oldenburg SJ, Monteiro-Riviere NA. Evaluation of silver nanoparticle toxicity in skin in vivo and keratinocytes in vitro. Environ Health Perspect. 2010;118(3):407–413. doi: 10.1289/ehp.0901398.

[72] Zhang XF, Shen W, Gurunathan S. Silver nanoparticle-mediated cellular responses in various cell lines: An in vitro model. Int J Mol Sci. 2016;17(10):1603. doi: 10.3390/ijms17101603.
[73] Brassolatti P, de Almeida Rodolpho JM, Franco de Godoy K, et al. Functionalized titanium nanoparticles induce oxidative stress and cell death in human skin cells. Int J Nanomed. 2022;17:1495–1509. doi: 10.2147/IJN.S325767.
[74] Park Y-H, Jeong SH, Yi SM, et al. Analysis for the potential of polystyrene and TiO 2 nanoparticles to induce skin irritation, phototoxicity, and sensitization. Toxicol In Vitro. 2011;25(8):1863–1869. doi: 10.1016/j.tiv.2011.05.022.
[75] Fusco L, Garrido M, Martín C, et al. Skin irritation potential of graphene-based materials using a non-animal test. Nanoscale. 2020;12(2):610–622. doi: 10.1039/c9nr06815e.
[76] Skin corrosion/irritation. International Labour Organization.
[77] Sanches PL, Geaquinto LRdO, Cruz R, et al. Toxicity evaluation of tio2 nanoparticles on the 3D skin model: A systematic review. Front Bioeng Biotechnol. 2020;8:575. doi: 10.3389/fbioe.2020.00575.
[78] Choi J, Kim H, Choi J, et al. Skin corrosion and irritation test of sunscreen nanoparticles using reconstructed 3D human skin model. Environ Health Toxicol. 2014;29:e2014004. doi: 10.5620/eht.2014.29.e2014004.
[79] Liu Y, Li N, Chen L, et al. A ready-to-use integrated in vitro skin corrosion and irritation testing strategy using EpiSkin™ model in China. Toxicol In Vitro. 2020;65:104778. doi: 10.1016/j.tiv.2020.104778.
[80] Liu W, Zhou W, Liu S, et al. Electrical impedance performance of metal dry bioelectrode with different surface coatings. Sens Actuators A Phys. 2018;269:515–523. doi: 10.1016/j.sna.2017.12.006.
[81] Carlin M, Garrido M, Sosa S, et al. In vitro assessment of skin irritation and corrosion properties of graphene-related materials on a 3D epidermis. Nanoscale. 2023;15(35):14423–14438. doi: 10.1039/d3nr03081d.
[82] Kim SY, Seo S, Choi KH, et al. Evaluation of phototoxicity of tattoo pigments using the 3 T3 neutral red uptake phototoxicity test and a 3D human reconstructed skin model. Toxicol In Vitro. 2020;65:104813. doi: 10.1016/j.tiv.2020.104813.
[83] Uco DP, Leite-Silva VR, Silva HDT, et al. UVA and UVB formulation phototoxicity in a three-dimensional human skin model: photodegradation effect. Toxicol In Vitro. 2018;53:37–44. doi: 10.1016/j.tiv.2018.07.009.
[84] Helena K, Liebsch M. The EpiDermTM phototoxicity test (EpiDermTM H3D-PT). In Alternatives for dermal toxicity testing. Springer; 2017. p. 1–592. doi: 10.1007/978-3-319-50353-0.
[85] MacKay C, Davies M, Summerfield V, et al. From pathways to people: Applying the adverse outcome pathway (AOP) for skin sensitization to risk assessment. Altex. 2013;30(4):473–486. doi: 10.14573/altex.2013.4.473.
[86] Gądarowska D, Kalka J, Daniel-Wójcik A, et al. Alternative methods for skin-sensitization assessment. Toxics. 2022;10(12):1–25. doi: 10.3390/toxics10120740.
[87] Guyard-Nicodème M, Gerault E, Platteel M, et al. Development of a multiparametric in vitro model of skin sensitization. J Appl Toxicol. 2015;35(1):48–58. doi: 10.1002/jat.2986.
[88] Neves CR, Gibbs S. Progress on reconstructed human skin models for allergy research and identifying contact sensitizers, vol. 37, no. October. Springer International Publishing; 2012. doi: 10.1007/82.
[89] Saito K, Nukada Y, Takenouchi O, et al. Development of a new in vitro skin sensitization assay (Epidermal Sensitization Assay; EpiSensA) using reconstructed human epidermis. Toxicol In Vitro. 2013;27(8):2213–2224. doi: 10.1016/j.tiv.2013.08.007.
[90] Zareba G, Cernichiari E, Hojo R, et al. Thimerosal distribution and metabolism in neonatal mice. J Appl Toxicol. 2007;27(5):511–518. doi: 10.1002/jat.
[91] Svobodová L, Rucki M, Vlkova A, et al. Sensitization potential of medical devices detected by in vitro and in vivo methods. Altex. 2021;38(3):419–430. doi: 10.14573/altex.2008142.
[92] UE. Commission regulation (EU) 2016/1688 of 20 September 2016 amending Annex VII to Regulation (EC) No 1907/2006 of the European Parliament and of the council on the Registration. Off J Eur Union. 2016;1688(440):30–32.
[93] Chen L, Li N, Liu Y, et al. A new 3D model for genotoxicity assessment: EpiSkin™ micronucleus assay. Mutagenesis. 2021;36(1):51–61. doi: 10.1093/mutage/geaa003.
[94] Kirkland D, Aardema M, Henderson L, et al. Evaluation of the ability of a battery of three in vitro genotoxicity tests to discriminate rodent carcinogens and non-carcinogens: I. Sensitivity, specificity and relative predictivity. Mutat Res. 2005;584(1–2):1–256. doi: 10.1016/j.mrgentox.2005.02.004.
[95] Roy S, Kulkarni R, Hewitt NJ, et al. The EpiDerm TM 3D human reconstructed skin micronucleus (RSMN) assay: historical control data and proof of principle studies for mechanistic assay adaptations. Mutat Res Genet Toxicol Environ Mutagen. 2016;805:25–37. doi: 10.1016/j.mrgentox.2016.05.010.
[96] Pfuhler S, Downs TR, Hewitt NJ, et al. Original manuscript editor ' s choice validation of the 3D reconstructed human skin micronucleus (RSMN) assay : an animal-free alternative for following-up positive results from standard in vitro genotoxicity assays. Mutagenesis. 2021;36(1):1–17. doi: 10.1093/mutage/geaa035.
[97] Flamand N, Marrot L, Belaidi J, et al. Development of genotoxicity test procedures with Episkin ®, a reconstructed human skin model : Towards new tools for in vitro risk assessment of dermally applied compounds. Mutat Res. 2006;606(1-2):39–51. doi: 10.1016/j.mrgentox.2006.02.009.
[98] Reisinger K, et al. Validation of the 3D Skin Comet assay using full thickness skin models : Transferability and reproducibility. Mutat Res Genet Toxicol Environ Mutagen. 2018;827:27–41. doi: 10.1016/j.mrgentox.2018.01.003.
[99] Genotoxicity testing using 3D models. Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment. 2024
[100] Ali N, Hosseini M, Vainio S, et al. Skin equivalents: skin from reconstructions as models to study skin development and diseases. Br J Dermatol. 2015;173(2):391–403. doi: 10.1111/bjd.13886.
[101] Tan SH, Chua DAC, Tang JRJ, et al. Design of hydrogel-based scaffolds for in vitro three-dimensional human skin model reconstruction. Acta Biomater. 2022;153:13–37. doi: 10.1016/j.actbio.2022.09.068.
[102] Hofmann E, Schwarz A, Fink J, et al. Modelling the complexity of human skin in vitro. Biomedicines. 2023;11(3):794. doi: 10.3390/biomedicines11030794.
[103] Ponec M, Boelsma E, Weerheim A, et al. Lipid and ultrastructural characterization of reconstructed skin models. Int J Pharm. 2000;203(1-2):211–225. doi: 10.1016/S0378-5173(00)00459-2.
[104] Dabrowska AK, Rotaru GM, Derler S, et al. Materials used to simulate physical properties of human skin. Skin Res Technol. 2016;22(1):3–14. doi: 10.1111/srt.12235.
[105] Amirrah IN, Lokanathan Y, Zulkiflee I, et al. A comprehensive review on collagen type I development of biomaterials for tissue engineering: from biosynthesis to bioscaffold. Biomedicines. 2022;10(9):2307. doi: 10.3390/biomedicines10092307.
[106] Randall MJ, Jüngel A, Rimann M, et al. Advances in the biofabrication of 3D skin in vitro: Healthy and pathological models. Front Bioeng Biotechnol. 2018;6:154. doi: 10.3389/fbioe.2018.00154.
[107] Vidal SEL, Tamamoto KA, Nguyen H, et al. 3D biomaterial matrix to support long term, full thickness, immuno-competent human skin equivalents with nervous system components. Biomaterials. 2019;198:194–203. doi: 10.1016/j.biomaterials.2018.04.044.
[108] Morales-Hurtado M, Zeng X, Gonzalez-Rodriguez P, et al. A new water absorbable mechanical Epidermal skin equivalent: The combination of hydrophobic PDMS and hydrophilic PVA hydrogel. J Mech Behav Biomed Mater. 2015;46:305–317. doi: 10.1016/j.jmbbm.2015.02.014.
[109] Tahri S, Maarof M, Masri S, et al. Human epidermal keratinocytes and human dermal fibroblasts interactions seeded on gelatin hydrogel for future application in skin in vitro 3-dimensional model. Front Bioeng Biotechnol. 2023;11:1200618. doi: 10.3389/fbioe.2023.1200618.
[110] Rosa E, Diaferia C, Gianolio E, et al. Multicomponent Hydrogel matrices of Fmoc-FF and cationic peptides for application in tissue engineering. Macromol Biosci. 2022;22(7):e2200128. doi: 10.1002/mabi.202200128.

[111] Burke G, Barron V, Geever T, et al. Evaluation of the materials properties, stability and cell response of a range of PEGDMA hydrogels for tissue engineering applications. J Mech Behav Biomed Mater. 2019;99:1–10. doi: 10.1016/j.jmbbm.2019.07.003.
[112] Khan J, Rudrapal M, Bhat EA, et al. Perspective insights to bio-nanomaterials for the treatment of neurological disorders. Front Bioeng Biotechnol. 2021;9:724158. doi: 10.3389/fbioe.2021.724158.
[113] Somasekharan LT, Raju R, Kumar S, et al. Biofabrication of skin tissue constructs using alginate, gelatin and diethylaminoethyl cellulose bioink. Int J Biol Macromol. 2021;189:398–409. doi: 10.1016/j.ijbiomac.2021.08.114.
[114] Sahu N, Gupta D, Nautiyal U. Hydrogel: preparation, characterization and applications. Asian Pac J Nurs Health Sci. 2020;3(1):1–11.
[115] Choudhury S, Das A. Advances in generation of three-dimensional skin equivalents: pre-clinical studies to clinical therapies. Cytotherapy. 2021;23(1):1–9. doi: 10.1016/j.jcyt.2020.10.001.
[116] Ravindran Girija A, Palaninathan V, Strudwick X, et al. Collagen-functionalized electrospun smooth and porous polymeric scaffolds for the development of human skin-equivalent. RSC Adv. 2020;10(45):26594–26603. doi: 10.1039/d0ra04648e.
[117] Cavallo A, Al Kayal T, Mero A, et al. Fibrinogen-based bioink for application in skin equivalent 3D bioprinting. J Funct Biomater. 2023;14(9):459. doi: 10.3390/jfb14090459.
[118] Black AF, Bouez C, Perrier E, et al. Optimization and characterization of an engineered human skin equivalent. Tissue Eng. 2005;11(5-6):723–733. doi: 10.1089/ten.2005.11.723.
[119] Berghard A, Gradin K, Toftgård R. Serum and extracellular calcium modulate induction of cytochrome P-450IA1 in human keratinocytes. J Biol Chem. 1990;265(34):21086–21090. doi: 10.1016/s0021-9258(17)45330-0.
[120] Bloom N, Van Reenen J. Human resource management and productivity. NBER Working Papers 16019. Available from: http://www.nber.org/papers/w16019
[121] Ouwehand K, Spiekstra SW, Waaijman T, et al. Technical advance: Langerhans cells derived from a human cell line in a full-thickness skin equivalent undergo allergen-induced maturation and migration. J Leukoc Biol. 2011;90(5):1027–1033. doi: 10.1189/jlb.0610374.
[122] Griffoni C, Neidhart B, Yang K, et al. In vitro skin culture media influence the viability and inflammatory response of primary macrophages. Sci Rep. 2021;11(1):7070. doi: 10.1038/s41598-021-86486-7.
[123] Sriram G, Alberti M, Dancik Y, et al. Full-thickness human skin-on-chip with enhanced epidermal morphogenesis and barrier function. Mater Today. 2018;21(4):326–340. doi: 10.1016/j.mattod.2017.11.002.
[124] Dixit S, Baganizi DR, Sahu R, et al. Immunological challenges associated with artificial skin grafts: Available solutions and stem cells in future design of synthetic skin. J Biol Eng. 2017;11(1):49. doi: 10.1186/s13036-017-0089-9.
[125] O'Neill AT, Monteiro-Riviere NA, Walker GM. Characterization of microfluidic human epidermal keratinocyte culture. Cytotechnology. 2008;56(3):197–207. doi: 10.1007/s10616-008-9149-9.
[126] Sidhaye VK, Schweitzer KS, Caterina MJ, et al. Shear stress regulates aquaporin-5 and airway epithelial barrier function. Proc Natl Acad Sci U S A. 2008;105(9):3345–3350. doi: 10.1073/pnas.0712287105.
[127] Yan H, Tang H, Qiu W, et al. A new dynamic culture device suitable for rat skin culture. Cell Tissue Res. 2019;375(3):723–731. doi: 10.1007/s00441-018-2945-4.
[128] Strickland JC. Guide to research techniques in neuroscience. J Undergrad Neurosci Educ. 2014;13(1):R1–2.
[129] Philippeos C, Hughes RD, Dhawan A, et al. Introduction to cell culture. In Human cell culture protocols, methods in molecular biology. Springer; 2012. p. 301–336. doi: 10.1007/978-1-61779-367-7.
[130] Naomi R, Bahari H, Ridzuan PM, et al. Natural-based biomaterial for skin wound healing (Gelatin vs. collagen): Expert review. Polymers (Basel). 2021;13(14):2319. doi: 10.3390/polym13142319.
[131] Ullah S, Zainol I, Chowdhury SR, et al. Development of various composition multicomponent chitosan/ fish collagen/glycerine 3D porous scaffolds: effect on morphology, mechanical strength, biostability and cytocompatibility. Int J Biol Macromol. 2017;111:158–168. doi: 10.1016/j.ijbiomac.2017.12.136.
[132] Widgerow AD. Bioengineered matrices-part 1: Attaining structural success in biologic skin substitutes. Ann Plast Surg. 2012;68(6):568–573. doi: 10.1097/SAP.0b013e31824b3d04.
[133] Fayyazbakhsh F, Leu MC. A brief review on 3D bioprinted skin substitutes. Procedia Manuf. 2020;48:790–796. doi: 10.1016/j.promfg.2020.05.115.
[134] Niehues H, Bouwstra JA, El Ghalbzouri A, et al. 3D skin models for 3R research: the potential of 3D reconstructed skin models to study skin barrier function. Exp Dermatol. 2018;27(5):501–511. doi: 10.1111/exd.13531.
[135] Suhail S, Sardashti N, Jaiswal D, et al. Engineered skin tissue equivalents for product evaluation and therapeutic applications. Biotechnol J. 2019;14(7):e1900022. doi: 10.1002/biot.201900022.
[136] Grimm H, Biller-Andorno N, Buch T, et al. Advancing the 3Rs: innovation, implementation, ethics and society. Front Vet Sci. 2023;10:1185706. doi: 10.3389/fvets.2023.1185706.
[137] Wilm A, Stork C, Bauer C, et al. Skin doctor : machine learning models for skin sensitization prediction that provide estimates and indicators of prediction reliability. IJMS. 2019;20(19):4833. doi: 10.3390/ijms20194833.
[138] Jimenez-Carretero D, Abrishami V, Fernández-de-Manuel L, et al. Tox_(R)CNN: deep learningbased nuclei profiling tool for drug toxicity screening. PLoS Comput Biol. 2018;14(11):e1006238. doi: 10.1371/journal.pcbi.1006238.
[139] Hu F, Santagostino SF, Danilenko DM, et al. Assessment of skin toxicity in an in vitro reconstituted human epidermis model using deep learning. Am J Pathol. 2022;192(4):687–700. doi: 10.1016/j.ajpath.2021.12.007.
[140] Shin J, Lee Y, Li Z, et al. Optimized 3D bioprinting technology based on machine learning : a review of recent trends and advances. Micromachines. 2022;13(3):363.
[141] Ng WL, Chan A, Ong YS, et al. Deep learning for fabrication and maturation of 3D bioprinted tissues and organs. Virtual Phys Prototyping. 2020;15(3):340–358. doi: 10.1080/17452759.2020.1771741.
[142] Jin Z, Zhang Z, Shao X, et al. Monitoring anomalies in 3D bioprinting with deep neural networks. ACS Biomater Sci Eng. 2021:9(7):3945–3952. doi: 10.1021/acsbiomaterials.0c01761.
[143] Ruberu K, Senadeera M, Rana S, et al. Coupling machine learning with 3D bioprinting to fast track optimisation of extrusion printing. Appl Mater Today. 2021;22:100914. doi: 10.1016/j.apmt.2020.100914.
[144] Villeneuve DL, Crump D, Garcia-Reyero N, et al. Adverse outcome pathway (AOP) development I : strategies and principles. Toxicol Sci. 2014;142(2):312–320. doi: 10.1093/toxsci/kfu199.
[145] Richard AM, Judson RS, Houck KA, et al. The ToxCast chemical landscape: paving the road to 21st century toxicology. Chem Res Toxicol. 2016;29(8):1225–1251. doi: 10.1021/acs.chemrestox.6b00135.
[146] Dix DJ, Houck KA, Martin MT, et al. The ToxCast program for prioritizing toxicity testing of environmental chemicals. Toxicol Sci. 2007;95(1):5–12. doi: 10.1093/toxsci/kfl103.
[147] Yang H, Sun L, Li W, et al. In silico prediction of chemical toxicity for drug design using machine learning methods and structural alerts. Front Chem. 2018;6:30. doi: 10.3389/fchem.2018.00030.
[148] Kang Y, Gyu M, Kyung K, et al. Machine - learning based prediction models for assessing skin irritation and corrosion potential of liquid chemicals using physicochemical properties by XGBoost. Toxicol Res. 2023;39(2):295–305. doi: 10.1007/s43188-022-00168-8.
[149] Roggen EL. In vitro toxicity testing in the twenty-first century. Front Pharmacol. 2011;2:3. doi: 10.3389/fphar.2011.00003.
[150] OECD . Guidance document on an integrated approach on testing and assessment (IATA) for skin corrosion and irritation. OECD Series on Testing and Assessment, No. 203, OECD Publishing, Paris. 2017.
[151] NTP. Alternative Methods Accepted by US Agencies. 2021; pp. 1–5. https://Ntp.niehs.nih.gov/pubhealth/evalatm/accept-methods/index.html. [accessed December 17, 2019]. Available at: https://ntp.niehs.nih.gov/whatwestudy/niceatm/accept-methods/index.html?utm_source=direct&utm_medium=prod&utm_campaign=ntpgolinks&utm_term=regaccept.

[152] Sun T, Jackson S, Haycock JW, et al. Culture of skin cells in 3D rather than 2D improves their ability to survive exposure to cytotoxic agents. J Biotechnol. 2006;122(3):372–381. doi: 10.1016/j.jbiotec.2005.12.021.

[153] Zhang Z, Michniak-Kohn BB. Tissue engineered human skin equivalents. Pharmaceutics. 2012;4(1):26–41. doi: 10.3390/pharmaceutics4010026.

[154] Akagi T, Nagura M, Hiura A, et al. Construction of three-dimensional dermo-epidermal skin equivalents using cell coating technology and their utilization as alternative skin for permeation studies and skin irritation tests. Tissue Eng Part A. 2017;23(11-12):481–490. doi: 10.1089/ten.tea.2016.0529.

[155] Sachana M, Hargreaves AJ. Toxicological testing. In Vivo and in vitro models. 3rd ed. Elsevier Inc; 2018. doi: 10.1016/B978-0-12-811410-0.00009-X.

[156] Auletta CS. Current in vivo assays for cutaneous toxicity: local and systemic toxicity testing. Basic Clin Pharmacol Toxicol. 2004;95(5):201–208. doi: 10.1111/j.1742-7843.2004.pto950501.x.

[157] Chipangura JK, Ntamo Y, Mohr B, et al. A review of challenges and prospects of 3D cell-based culture models used for studying drug induced liver injury during early phases of drug development. Hum Exp Toxicol. 2023;42:9603271221147884. doi: 10.1177/09603271221147884.

[158] Picollet-D'hahan N, Zuchowska A, Lemeunier I, et al. Multiorgan-on-a-chip: A systemic approach to model and decipher inter-organ communication. Trends Biotechnol. 2021;39(8):788–810. doi: 10.1016/j.tibtech.2020.11.014.

[159] Schmeisser S, Miccoli A, von Bergen M, et al. New approach methodologies in human regulatory toxicology – not if, but how and when. Environ Int. 2023;178:108082. doi: 10.1016/j.envint.2023.108082.

[160] OECD. OECD guidance on the reporting of defined approaches and individual information sources to be used within Integrated Approaches to Testing and Assessment (IATA) for skin sensitization. 2016. doi: 10.1016/j.toxlet.2016.06.027.