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<th>3D bioprinting of skin constructs for toxicology testing</th>
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ABSTRACT: The high discrepancies between adverse effects of chemicals in animal and human have led to the development of alternative in-vitro human tissue models to improve the reliability and accuracy of toxicology testing. 3D bioprinting technology has emerged as an advanced platform that facilitates simultaneous and highly-specific patterning of multiple types of cells and biomaterials, which is lacking in conventional tissue engineering approaches. The goal of this review is to highlight the achievements in skin bioprinting and present the future outlook on standardization of in-vitro human tissue models for toxicology testing.

KEYWORDS: 3D printing, 3D bioprinting, skin tissue engineering, human tissue models, toxicology testing

1. Toxicology testing

Toxicology testing is performed to identify the potential adverse effects a chemical poses to an individual and its surrounding environment (Hartung, 2009b). The different types of chemicals include active pharmaceutical drugs, cosmetics ingredients, household and industrial chemicals. An estimated number of 2,000 new chemicals are produced annually for various applications; routine toxicology tests are conducted on increasing number of new chemicals on a daily basis to ensure its safety to potential consumers. The global market for in-vitro toxicology testing market has been estimated to be ~USD 13 billion in 2016 and it is projected to reach USD 20.8 billion by the end of 2021 (MarketsandMarkets, 2016). An ideal study to evaluate the toxicity of a chemical/substance to humans would require an extremely large number of human subjects who are representative of the diversity of humans, which is unrealistic and unethical. As such, the use of animal models provides preliminary safety data to satisfy the conservative regulatory requirements. The crucial issue is the extent to which these animal models can predict the human responses in an accurate and reliable manner. It is clearly evident that the use of animal models has several caveats: the differences in the absorption or distribution of the chemicals/substances; the way the substances are metabolized and the short duration of animal lifespan (to accurately monitor disease development). As such, the use of animal models remains highly controversial as there are significant discrepancies between adverse effects of chemicals in humans and animals (Lilienblum et al., 2008). Furthermore, a complete ban on animal testing for cosmetics ingredients in 2013 has necessitates the development of alternative in-vitro skin models (W. L. Ng, Wang, Yeong, & Naing, 2016). A paradigm shift in the testing models has occurred over the last few years; the implementation of non-animal testing strategy has spurred the development of numerous 3D in-vitro testing methods (Burden, Sewell, & Chapman, 2015; Hartung, 2009a). In this review, we present and discuss the recent progress in skin bioprinting for potential toxicology applications.
2. 3D bioprinting approaches

The field of bioprinting has advanced tremendously over the last few years; it is a highly-automated fabrication platform that facilitates the pre-defined patterning of living cells, biomaterials and proteins via a layer-by-layer manufacturing process to print complex 3D tissue constructs with high throughput rates and reliability. 3D bioprinting has evolved into an advanced platform for fabrication of several tissues and/or organs such as skin (W. L. Ng, Yeong, W. Y. & Naing, M. W., 2014), heart tissue (Jana & Lerman, 2015), bone (Bosc, Vahabzadeh, & Bandyopadhyay, 2013), liver (Ikegami & Maehara, 2013), neural tissues (Zhuang, Sun, An, Chua, & Chew, 2018) and cartilage tissues (Cui, Breitenkamp, Finn, Lotz, & D’Lima, 2012) that offer radical solutions for prevailing healthcare problems. The field of 3D bioprinting can be classified under two main printing approaches; namely the drop-on-demand (DOD) printing (Gudupati, Dey, & Ozbolat, 2016; Lothar Koch, Brandt, Dietwick, & Chichkov, 2017; L. Koch, Gruene, Unger, & Chichkov, 2013; W. L. Ng, Lee, Yeong, & Win Naing, 2017; W. L. Ng, Yeong, & Naing, 2017; Saunders & Derby, 2014) (microvalve-based, inkjet-based and laser-based) and continuous printing (Ozbolat & Hospodiuk, 2016; Sunthornpong, Tan, An, & Chua, 2017) (extrusion-based). The key advantages of 3D printing approach over the conventional tissue engineering approach include its precise printing of living cells and/or biomaterials and subtle control of material volume at pre-defined positions to fabricate highly-complex tissue constructs (Xu et al., 2013). To date, 3D bioprinting systems have attracted tremendous attention for tissue engineering and regenerative medicine applications such as high-throughput drug screening for toxicology studies, fundamental cell biology research, fabrication of in-vitro tissue models and even in-situ bioprinting of cells and extracellular matrix for wound regeneration (Murphy & Atala, 2014). Most importantly, it offers standardization in the fabrication of human tissue models in a highly repeatable and reliable manner.

3. Current state-of-the-arts for 3D bioprinted skin constructs

![Diagram](https://via.placeholder.com/150)

Fig 1. The human skin cells are first expanded in-vitro to obtain a sufficient amount of cells prior to bioprinting and maturation of 3D bio-printed constructs. Reproduced with permission (W. L. Ng et al., 2016).
3.1. Microvalve-based bioprinting

Multi-layered collagen constructs containing human keratinocyte and fibroblast cell lines were deposited on a non-planar surface via layer-by-layer manufacturing approach (W. Lee et al., 2009) and greater cell viability in the 3D constructs with fluidic channels (85% viability) as compared to the ones without any channels (60% viability) was reported (W. Lee et al., 2010). A recent work emulated the native cellular density of different skin cells (HFF-1 fibroblasts and HaCaT keratinocytes) within the 3D bioprinted skin constructs using contactless microvalve print-heads (V. Lee et al., 2013) but the use of human skin cell lines resulted in poor stratification and keratinization of the printed keratinocyte layers.

As such, recent works on skin bioprinting utilized primary human stem cells and skin cells. The amniotic fluid-derived stem cells (AFSCs at a cell density of 16.6 x 10^6 cells/ml) were encapsulated in the fibrinogen/collagen solution at 0°C. Microvalve-based print-heads were used to print the fibrinogen/collagen solution and its cross-linker, thrombin, over the full-thickness skin wounds (2 cm x 2 cm) of nu/nu mice (Skardal et al., 2012). Although the AFSCs only remained transiently in wound sites, the secretion of important growth factors from AFSCs expedited wound closure rates and angiogenesis. Furthermore, the feasibility of in-situ printing was also explored on full thickness large wounds (10 cm x 10 cm) of nude mice (Binder et al., 2010). A single layer of fibrinogen/collagen hydrogel precursor containing fibroblasts (1.0 x 10^5 cells/cm^2) was first cross-linked by nebulized thrombin to form fibrin/collagen hydrogel, followed by bioprinting another layer of keratinocyte cells (1.0 x 10^7 cells/cm^2) above the fibroblast-populated fibrin/collagen matrix. Although complete re-epithelialization of the large wound was achieved after 8 weeks, it is highly challenging to deposit cells on areas of significant curvature. Notably, a recent study has reported the fabrication of pigmented human skin constructs (W. L. Ng, Tan, Yeong, & Naing, 2018). A two-step bioprinting strategy was implemented; hierarchical porous collagen-fibroblast dermal matrices were first fabricated using macromolecular crowding (W. L. Ng, Goh, Yeong, & Naing, 2018), followed by pre-defined patterning of the primary human keratinocytes and melanocytes in highly-specific arrangement found in native human skin. The 3D bioprinted human skin constructs were then matured under optimal culture conditions to eventually achieve the 3D pigmented human skin constructs. The 3D printed human skin constructs in most studies showed high resemblance to native skin structure and they can serve as potential in-vitro tissue models for toxicological testing.

3.2. Laser-based bioprinting

A recent in-vitro study deposited 20 layers of fibroblasts (mouse NIH-3T3) and subsequent 20 layers of keratinocytes (human HaCaT) embedded in collagen gel onto a sheet of Matriderm® (decellularized dermal matrix) (Lothar Koch et al., 2012). The presence of cadherins and connexin 43 (Cx 43) in the epidermis indicated tissue morphogenesis and cohesion. Another study reported good graft-take of printed skin construct with the surrounding tissue and angiogenesis from the wound bed was observed after 11 days of transplantation (Michael et al., 2013).
3.3. Extrusion-based bioprinting

A recent work demonstrated that the presence of a PCL mesh (serves as a functional transwell system) helped to stabilize the collagen-based dermal matrix and prevent severe contraction during the maturation process (Kim, Lee, Gao, & Cho, 2017). Primary human fibroblasts and keratinocytes obtained from skin biopsies of healthy donors were printed directly to fabricate fibrin-based skin constructs using extrusion-based printing approach. The plasma-derived fibrinogen matrix was first crosslinked with the calcium chloride (CaCl₂) solution to form the 3D fibrin-based dermal constructs populated with human fibroblasts (Cubo, Garcia, del Cañizo, Velasco, & Jorcano, 2016). The histological and immune-histochemical analysis showed that the 3D printed skin constructs is highly similar to the native human skin.
4. Future Outlook

One of the greatest challenges in toxicological testing will be the need to standardize the existing testing models. Currently, many researchers are developing 3D organotypic cultures that closely mimic the skin structure and function (Visk, 2015). As such, it is important to evaluate and assess the current in-vitro skin models and their limitations. Furthermore, it is also important to evaluate the inter-laboratory reproducibility of such in-vitro skin models. The implementation of 3D printing technology can offer a highly-automated fabrication process for standardization of in-vitro testing models. To date, no single testing approach is perfect and it is important to combine different testing approaches and integrate them into a holistic testing strategy to enhance the accuracy and reliability of toxicological testing.

5. Concluding Remarks

The bioprinting platform offers high spatial control over the cellular and material deposition which is critical for cell-cell and cell-matrix interactions. To date, several researchers have demonstrated the fabrication of biomimetic skin constructs using different bioprinting techniques and the implementation of 3D bioprinting techniques not only helps to standardize the fabrication of in-vitro skin models but also improves the inter-laboratory reproducibility of in-vitro skin models.

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