3D Bioprinting Skin and Melanoma Models

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Melanoma is a potentially fatal cancer with rising incidence, associated with enhanced sun exposure and ultraviolet radiation. Its incidence is highest in people of European descent and the ageing population. Although survival has improved due to advances in targeted and immunotherapies, new understanding of melanoma biology and disease progression is vital to improving clinical outcomes. Efforts to develop three-dimensional human skin equivalent models using biofabrication techniques, such as bioprinting, promise to deliver a better understanding of the complexity of melanoma and associated risk factors. These 3D skin models can be used as a platform for patient specific models and testing therapeutics.

Keywords: 3D printing ; bioprinting ; disease models ; melanoma

1. Introduction

The interest in using bioprinting to fabricate artificial skin has significantly increased in the last decade. This has been mainly motivated by the unprecedented ability of these technologies in depositing cells, biomaterials, and bioactive molecules in predefined 3D locations with high precision and reproducibility [1][2][3]. Bioprinted HSEs, exhibiting varying levels of biological function and complexity at structural, material, and cellular level, has been fabricated using different bioprinting technologies including vat polymerisation [4][5], inkjet [6][7][8][9], laser-assisted [10][11], and extrusion [6][12][13][14][15][16][17]. In vitro models of skin have experienced important advances as demonstrated by the bioprinting of multilayer tissue constructs comprising multiple cells, such as fibroblasts, endothelial cells, adipocytes, pericytes, stem cells, induced pluripotent stem cells (iPSCs), melanocytes, and keratinocytes [7][14][18][19][20][21].

A major advantage of bioprinting relies on the ability to closely mimic the architecture of the native skin through the deposition of bioinks comprised of skin cells and bioinstructive materials into a 3D construct with a tissue-specific organisation. It has been demonstrated that increasing the complexity and biomimicry of bioprinted skin translates to improved biological function, predictive value, and healing ability [16][18]. The feasibility of skin bioprinting has been demonstrated not only in vitro but also in situ by pre-clinical studies showing that the deposition of cells (stem cells, fibroblasts and/or keratinocytes) directly onto the wound bed of mouse or porcine models stimulates healing [22][23]. Although bioprinted in vitro skin has primarily been used as grafts for skin repair, a great interest is now focused on creating advanced tissue models capable of modelling key biophysical, biochemical, and biological features of both healthy and diseased skin. Such models are appealing and urgently needed as platforms for the screening and validation of drugs and cosmetics, as well as to perform fundamental studies to unravel mechanisms underlying skin diseases. Efforts have also been made in creating 3D bioprinted models of skin diseases, such as atopic dermatitis [13], psoriasis [24], and cutaneous melanoma [25][26]. However, the reported achievements in bioprinting diseased skin models are more modest so far. The following sections provide an overview of the design considerations for bioinks, bioprinting technologies, and advancements in bioprinted skin models to provide the contextual background and understanding required to develop a bioprinted melanoma model. Such topics are reviewed in detail here [2][3][27][28][29][30][31][32][33][34][35][36][37][38][39][40][41][42][43].

2. Bioinks

The effective fabrication of a multicellular and structurally complex bioprinted HSE requires the use of a suitable bioink. A bioink is a cell formulation that can be processed using biofabrication technologies and that can optionally contain biomaterials (e.g., hydrogel precursors) and bioactive molecules (e.g., DNA and growth factors) [32]. In contrast, a biomaterial ink formulation has no cells and only encounters cells after fabrication, for example, through cell seeding. Both approaches, and their combination, have been explored in skin bioprinting.

A range of bioink design parameters must be considered during the development of a suitable bioink for skin bioprinting. Bioinks are typically composed of pre-crosslinked hydrogels or hydrogel precursors that are crosslinked during or post-

fabrication into 3D hydrogel networks [31][33][34][39][44][45][46]. Hydrogels are a popular class of materials for bioink design due to their hydrophilic nature, which allows for the absorption and retention of large amounts of water, and resemblance to the hydrated state of native ECM. The biomaterial selection and crosslinking reaction are crucial in determining the bioink biocompatibility (e.g., cell viability, adhesion, proliferation, and differentiation), printability (e.g., rheology and shape fidelity), biomechanics (e.g., resemblance to target tissue), and biodegradation (e.g., match rate of tissue formation). For example, the selection of the optimal biomaterials for a bioink is a significant challenge. Natural biomaterials are typically biocompatible, biodegradable, versatile and can exhibit native cell binding and instructive motifs (animal derived biomaterials) [33][34][36][39][42]. However, despite excellent biological properties, natural materials often have poor mechanical properties, which commonly translates into limited printability. Alternatively, synthetic biomaterials display superior mechanical properties and stability, which can be explored to improve printability. However, synthetic materials lack native cell instructive motifs thus limiting biocompatibility and biodegradation [33][34][39][41][47][48].

Subsequently, a range of approaches to modify specific bioink attributes have been explored to improve printability and biocompatibility [29][30][37][38][44][45][49]. Hydrogel precursors can be modified through the addition of functional groups, for example, to allow chemical crosslinking (e.g., gelatin functionalised with methacryloyl) or the incorporation of cell instructive motifs (e.g., RGD) to promote cell attachment. A myriad of chemical (e.g., enzyme catalysed reactions, photopolymerisation, and click chemistry) and physical (e.g., thermal, ionic, and hydrogen bonding) crosslinking schemes are nowadays utilised in bioink design to tune printability and cell response of hydrogels. Novel dynamic and responsive bioinks that can broaden the biofabrication window and provide a more biomimetic environment for cells are being developed [29][30][37][38][44][45][49].

However, single material bioinks can struggle to meet the requirements of both printability and biocompatibility, hence the development of multicomponent bioinks [29][30][37][38][44][45][49]. These include interpenetrating networks, nanocomposites, supramolecular, and multi-material dual-crosslinked hydrogels which enable improvements in printability and biological functionality.

3. 3D Bioprinting Technologies

Three-dimensional bioprinting encompasses a range of additive manufacturing technologies that can process bioinks into structurally complex and multi-material tissue constructs with high reproducibility. Bioprinting systems can comprise multiple printheads and technologies within a single machine working in tandem to allow multi-material printing. As the skin is a complex organ with a multicellular, multi-layered, and hierarchical organisation; the bioprinting technology selected should be able to replicate this architecture and be suitable for the specific bioink rheological properties. For skin bioprinting, the most relevant techniques are extrusion, material jetting (inkjet), laser assisted, and lithographic bioprinting.

Extrusion-based bioprinting comprises systems that use pneumatic (compressed air), mechanical (screw/piston), or solenoid driven printheads to deposit bioinks onto a platform [28][30][31][35][38][43][50][51][52][53]. The majority of skin bioprinting studies utilise extrusion-based printing. Extrusion-based techniques are versatile, simple, low-cost, and widely used as a broad range of bioink viscosities with high cell densities can be processed into structures with high vertical shape fidelity. The bioinks should exhibit shear thinning or thixotropic properties. In comparison to other bioprinting techniques, extrusion presents lower printing resolution and cell viability (40–80%). The slow printing speed, especially with highly viscous materials, and the resulting shear stresses experienced by the cells in the nozzle (decreasing nozzle diameter increases shear stress) can compromise cell viability. Furthermore, extrusion bioprinting has some limitations in terms of precisely managing the volume of printed material during the lag period between switching the pressure on and off with the added potential risk of the nozzle clogging. The flexibility of extrusion-based bioprinting is also due to the variety of printing strategies that can be used, such as the use of coagulation baths, support baths, and co-axial extrusion [54][55][56]. These strategies can broaden the types of bioinks used, enabling difficult to print biomaterials, such as collagen to be accessible, and allowing unique construct and fibre architectures to be printed that are not possible through conventional extrusion.

Material jetting, continuous inkjet (CI) or drop on demand (DOD), is a bioprinting technique that deposits discrete bioink droplets in a layer-by-layer process onto a platform [28][34][38][43][57][58][59][60]. DOD uses an actuator (thermal, acoustic, piezoelectric, valves) to generate droplets of a specific size. Alternatively, CI exploits the Plateau–Raleigh instability of a flowing fluid to form discrete droplets. Material jetting is a low-cost technique with high print speed, resolution (20–100 µm), and cell viability (>85%). The bioinks used should present rheopectic properties to minimise shape change of the droplet after deposition. Although material jetting has a high print resolution the vertical fidelity of printed structures is low. The process is limited to printing low viscosity materials (<10 mPa s) with low cell concentrations (<10^6 cells mL^{-1}), with the additional problem of nozzle clogging at high viscosities. Furthermore, acoustic and thermal actuators can impact cell viability due to the frequencies and temperatures used.

Laser-assisted bioprinting (LAB) consists of three main elements: a pulsed laser source, a donor slide (ribbon), and a collector [34][38][61][62][63][64][65]. The donor slide comprises a laser transparent support (glass) with a thin coating of an energy absorbing metal layer (e.g., gold or titanium) and a final bioink layer. Laser irradiation and absorption of the energy at the focal point in the metal layer induces local evaporation and formation of an expanding bubble that causes a shockwave and jet formation that ejects a bioink droplet onto the collector. As LAB is a nozzle-free printing process, clogging is not a limitation. Furthermore, LAB is characterised by high printing resolution (droplets 10–100 µm) and cell viability (>95%), as well as the ability to print individual cells with high spatiotemporal control. However, these systems are expensive limiting their utilisation. Furthermore, the heat produced by the laser irradiation and evaporation process can compromise cell viability, although advancements, such as absorbing-film assisted laser induced forward motion (LIFT) and matrix-assisted pulsed laser evaporation direct write (MAPLE-DW) have mitigated this. The bioinks used have a limited range of viscosities (1–300 mPa·s) and cell densities; they must adhere to the donor slide, and the printing process only allows for one bioink to be used at a time.

Lithographic bioprinting processes, such as stereolithography (SLA), digital light processing (DLP), and two photon polymerisation (2PP) use specific wavelengths and intensities of light to selectively polymerise a bioresin into a 3D structure [34][38][66]. The bioresin can consist of photoinitiators, reactive monomers/macromers, additives (e.g., photo absorbers and radical scavengers), and cells. Lithographic techniques generally have high resolution with DLP achieving ~25–50 µm, whilst 2PP has a significantly higher resolution of ~100 nm; however, the 2PP resolution comes at the cost of low print speed and small maximum size of the construct. Cell viability is typically high (>85%) and moderate cell densities ($<10^8$ cells mL^{-1}) have been utilised. However, cytocompatibility issues arising from the choice of photoinitiator, wavelength, and light intensity must be considered. Visible light and compatible photoinitiators are becoming an attractive alternative to traditional UV bioprinting due to the lower associated cytotoxicity [66]. The printing systems can be low-cost, especially commercially available printers adapted for bioprinting, however, specialised systems, such as 2PP are considerably more expensive. A low viscosity bioresin must be used to allow the unreacted resin to move out of the 3D structure during the printing process. This can cause issues with cell sedimentation, especially in constructs with long print times. An obvious limitation is that biomaterials are limited to photopolymerisable polymers, thus biomaterials must be modified to allow compatibility.

A new approach in lithographic bioprinting termed volumetric printing (also referred to as tomographic volumetric printing or computed axial lithography) can overcome some of the limitations of conventional bioprinting techniques [67][68][69]. This technique uses a rotating volume of bioresin that is selectively photopolymerised by a dynamic projection of 2D light patterns. This enables the fabrication of complex 3D structures within seconds to minutes in a non-layer-by-layer process. Overcoming long print times and associated cell viability issues, furthermore, the process is not restricted by conventional 3D printing structural limitations, such as support structures and z-layer lines.

4. Skin Models

Three-dimensional bioprinting has allowed the development of complex biomimetic models that begin to recapitulate the structure and composition of native skin. This is important as an advanced melanoma skin model should include epidermal, dermal, and hypodermal layers (full-thickness); appendages (e.g., hair follicles and sweat glands); pigmentation (melanocytes), and a functioning vasculature. The incorporation of all these into a single disease model is a major challenge. However, the complexity of understanding the melanoma TME, interactions with surrounding tissues, and disease progression require a complex and tuneable model. Nevertheless, despite the challenges significant progress has been made in the bioprinting of advanced skin models, which can be translated to cutting-edge models of melanoma [1][2][3].

The bioprinting of skin appendages, such as hair follicles and sweat glands is an important direction in the development of biomimetic skin models due to their role in thermoregulation, homeostatic maintenance, and wound healing. Abaci et al. [70] developed a hair follicle skin model using 3D printing to create a high-resolution mould to pattern microwells inside a collagen hydrogel encapsulated with fibroblasts. The microwells were seeded with dermal papilla cells, which spontaneously formed into aggregates, followed by the seeding of keratinocytes to create a hair follicle-like unit. In vitro results demonstrated differentiation of keratinocytes into specific hair lineages and prolonged culture even showed protrusion of hair follicles outside of the construct. In vivo engraftment of a vascularised construct, human umbilical vein endothelial cells encapsulated in the dermal layer showed human hair growth in a mouse model. Huang et al. [71] used extrusion-based bioprinting to develop a gelatin and alginate based bioink with epithelial progenitor cells, dermal homogenate, and epidermal growth factor. This aimed to recreate an inductive microenvironment to promote differentiation towards a sweat gland cell lineage. The results indicated that the bioink ECM-mimicking niche promoted sweat gland differentiation in vitro and in vivo was able to restore sweat gland functionality in a burn model. This approach

was further developed to directly differentiate mesenchymal stem cells into a sweat gland cell [72] Additionally, Zhang et al. [73] refined the model further by combining the bioprinted sweat gland construct with the seeding of hair follicle spheroids to create a construct with both appendages.

Skin pigmentation is a major aspect of developing a viable bioprinted melanoma model. Therefore, studies on bioprinting melanocytes and the evaluation of melanin production are crucial. For example, Ng et al. [7] exhibited a two-step DOD bioprinting technique to fabricate 3D pigmented skin constructs using a collagen and polyvinylpyrrolidone bioink containing keratinocytes, melanocytes, and fibroblasts. An initial dermal layer containing fibroblasts was printed and cultured prior to the precise deposition of melanocytes and keratinocytes droplets (1:8 ratio) and further cultured at the air–liquid interface. Histological analysis confirmed the presence of a mature stratified epidermis with uniform distribution of melanin. Similarly, Min et al. [21] printed a dermal structure consisting of multiple layers of collagen crosslinked with sodium bicarbonate and within these layers, a fibroblast bioink was embedded. Melanocytes were printed on top of the dermal layer either in a spot or full-layer pigmentation model followed by printing of keratinocytes and culturing at the air–liquid interface. The melanocytes showed dendritic formation in the epidermal layer and production and accumulation of melanin in a freckle-like morphology.

Vascularisation of skin constructs is important as the maximum gas and nutrient diffusion distance that can maintain cell survival is ~100–200 µm, hence, the long-term viability of the bioprinted skin construct relies on the creation of a functional vasculature [74][75]. Additionally, successful integration and viability of a HSE implant with the host tissue require either the implant is pre-vascularised and can rapidly integrate with the host tissue or is able to recruit and develop a vasculature via the host tissue. Furthermore, in melanoma a key mechanism for tumour growth and metastasis is via the vasculature, thus vascularised dermal and hypodermal components can provide a more in vivo-like environment that can promote cell–cell and cell–ECM interactions, vital for improved melanoma disease models. Using bioprinting, vascularised 3D HSEs can be engineered [5][19][76][77][78]. Yanez et al. [77] used inkjet bioprinting to fabricate a bilayer skin graft with human dermal microvascular endothelial cells embedded between a collagen dermal and epidermal layer containing fibroblasts and keratinocytes, respectively. The endothelial cell–thrombin bioink was printed into a fibrinogen layer to generate a fibrin vascular network. The in vivo study showed improved wound contraction, normal skin appearance, and the indication was that blood microvessels had formed and were integrating with the host tissue. An alternative approach by Abaci et al. [76] used 3D printing to create moulds to fabricate sacrificial alginate channels of specific vasculature patterns. Dermal and epidermal layers were cast on top of the vascular channels and the alginate was removed and the channels perfused with endothelial cells. The endothelial cells attached and coated the inner walls of the microchannels and exhibited appropriate barrier function. The vascularised HSEs promoted neovascularisation during wound healing in a mouse model.

As bioprinting technologies and bioink design have progressed, the ability to bioprint complex full-thickness HSE models has become possible [16][18][19][79][80][81]. These models include not only dermal and epidermal compartments, but additional aspects of skin, such as the hypodermis (trilayer), a vascular network, and appendages. This is a significant development in allowing the development of biologically relevant melanoma models. For example, Kim et al. [19] have developed a platform for the bioprinting of a full-thickness vascularised skin model that resembles native skin. The platform based on extrusion and inkjet printing, the use of multiple bioinks, a printed transwell support, perfusable vascular channels, and vascularised dermal and hypodermal layers provide a complex microenvironment that can be further developed to include additional skin appendages or interrogated as a disease model. Furthermore, Jorgensen et al. [16] bioprinted a full-thickness trilayer HSE containing keratinocytes and melanocytes (epidermal layer); fibroblasts, microvascular endothelial cells, and follicle dermal papillary cells (dermal layer) and preadipocytes (hypodermal layer). An extrusion-based bioprinter was used to print a fibrinogen based bioink crosslinked with thrombin to form a fibrin hydrogel. The bioprinted constructs were cultured for four days prior to implantation in a full-thickness mouse wound model. The bioprinted HSEs accelerated wound closure through enhanced epithelialisation and produced a normal basket-weave collagen matrix, potentially minimising scarring. The HSE after implantation resembled phenotypically human skin and was a mixture of both the implant and infiltrating host cells.

5. Bioprinting in Vitro Models of Melanoma

Despite existing skin and melanoma models having been proved to be useful for understanding disease pathology, performing drug screening, and even providing scientific support to clinical trials, a major challenge remains in the development of a melanoma model capable of better reproducing the different features of the tumour microenvironment underlying the in vivo resistance and differential responses in human patients. This is an ambitious and complex task as melanoma cells are characterised by high genetic instability and plasticity, and the ability to dedifferentiate into a variety of

states and secret factors that promote melanoma cell viability in an autocrine way and reprogram adjacent stroma cells that influence the tumour microenvironment in a paracrine manner [82].

The potential of bioprinting to create biomimetic melanoma models has been demonstrated in a few studies focused on the bioprinting of melanoma cells and evaluation of cell response. In a recent study, the influence of bioink properties and composition on the proliferation and morphology of metastasis-derived melanoma cell lines (Mel Im and MV3dc) was investigated [25]. Extrusion-based bioprinting was used and results showed that the printing fidelity, cell viability and proliferation were highly dependent on the bioink material. Although cells survived in bioprinted hydrogels, cell morphology, and proliferation were differentially affected by the tested bioinks owing to their distinct biophysical and biochemical properties. Despite the highest cell proliferation observed in Matrigel hydrogels, bioprinted constructs displayed poor shape fidelity and long-term stability, which limits the fabrication of 3D constructs with complex architectures. In a subsequent study, a more detailed characterisation was performed to correlate the properties (e.g., mechanical properties) of hydrogels (alginate, alginate dialdehyde crosslinked with gelatin, and thiol-modified hyaluronan crosslinked with polyethylene glycol diacrylate) with the response of melanoma cells, but using a manual approach to create the hydrogels instead of bioprinting [83]. Using a different strategy, extrusion-based bioprinting was explored to create collagen scaffolds to support the maintenance and survival of cryopreserved patient-derived melanoma explants seeded onto the bioprinted scaffolds [26]. The results showed improved cell maintenance and survival compared to standard 2D culture and retention of key melanoma biomarker expression.

Schmid et al. [84] developed a bioink consisting of gelatin, alginate, and hyaluronic acid (Alg/HA/Gel), which was successfully used for melanoma studies and mimics the tumour microenvironment. The results show that the developed bioink is suitable for extrusion-based bioprinting, providing good shape fidelity, and high cell survival post-printing. Moreover, in combination with an arteriovenous loop, the in vivo model provides a unique platform for studying melanoma progression, angiogenesis, and ultimately metastases similar to human pathophysiology, in an isolated and controlled environment. Furthermore, the bioink provides a highly defined material composition compared to other tumour models which rely on, for example, Matrigel which is a complex and poorly defined heterogenous basement membrane matrix derived from sarcoma cells that can have significant batch-to-batch differences in composition [85].

6. Challenges of Bioprinting Melanoma Models

Skin is a complex multi-functional tissue with anisotropic material, structural, and cellular properties. For example, the composition, density, and organisation of cells, appendages, and the ECM varies significantly between skin layers and within the different regions of the layers. Recreating this heterogeneity and architectural features, such as the wavy epidermal–dermal junction is a significant challenge in skin bioprinting.

This necessitates the development of enhanced software tools for designing and fabricating such complex constructs; advanced bioprinting systems for fabricating multi-material, hierarchical, and functionally graded skin constructs with high resolution and printing speed.

The selection of a bioink for a melanoma model will differ from a bioprinted skin graft. The specific requirement of each application means that the properties of the bioink will be distinct to provide specific microenvironmental cues to both normal and cancer cells. Skin grafts may favour long-term construct stability and promotion of host vasculature in-growth, but the bioink itself may not need to fully recapitulate the native ECM to fulfil its purpose of being a successful skin graft. Whilst a melanoma bioink should from the beginning as closely as possible resemble the native ECM to promote appropriate cell–ECM interactions and maintain markers of melanoma expression, which are crucial in understanding melanoma physiology and progression. Unmodified native skin ECM biomaterials, retaining all instructive and binding sequences, such as collagen may be the most desirable for this objective. Furthermore, with new bioprinting strategies, the difficulty of bioprinting collagen and other native polymers is no longer as much of a challenge [86][87][88].

The long-term viability of a bioprinted tissue requires the integration of a perfusable vascular network [75]. This is a key aim in tissue engineering to fabricate viable large-scale tissues and organs for implantation and integration with the host tissue. Additionally, the long-term study of biofabricated disease models is essential in developing a new understanding of pathophysiology and replacing equivalent animal models. This is especially important in cancer models due to the utilisation of the vasculature by tumour cells for angiogenesis, intravasation, metastasis, and circulation. Although vascularised melanoma skin models have not yet been developed, the tools and platforms are available and been demonstrated in bioprinted HSEs.

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