Development of pigmented human skin constructs via 3D drop-on-demand bioprinting

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**DEVELOPMENT OF PIGMENTED HUMAN SKIN
CONSTRUCTS VIA 3D DROP-ON-DEMAND
BIOPRINTING**

**NANYANG
TECHNOLOGICAL
UNIVERSITY**

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Development of Pigmented Human Skin Constructs via 3D Drop-on-Demand Bioprinting

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ABSTRACT

As a proof-of-concept, a two-step bioprinting strategy is implemented to fabricate the 3D pigmented human skin constructs. The first step involves the development and optimization of a suitable polyvinylpyrrolidone (PVP)-based bio-ink for printing of cells with enhanced viability and homogeneity. The experimental results have highlighted the importance of bio-ink properties (represented by Z values) on printed cells; the printed cells that are cultured over a period of 96-hours do not show significant printing-induced damage when the Z values of the PVP-based bio-inks are below 9.30. This critical step facilitates the patterning of epidermal melanin units (EMUs, cell-cell interactions between the keratinocytes and melanocytes) at an optimal ratio and density. The second step involves the engineering the complex 3D microstructures in the collagen-fibroblast matrices using macromolecular crowding. An in-depth investigation is performed to first understand the synergistic effect of macromolecular crowding (MMC) on the complex 3D collagen-fibroblast matrices. The MMC can be used to alter the 3D microstructures (pore size and porosity) within the collagen-fibroblast matrices; the pore size of the 3D collagen-fibroblast matrices plays a critical role in regulating the cell-matrix remodeling process and cellular behavior. The study highlights the importance of hierarchical pore sizes within the 3D dermal skin constructs and provides critical insights (optimal pore size range for each region of the proposed tri-zone dermal constructs) for the development of improved dermal skin constructs using MMC. As such, a drop-on-demand bioprinting-based strategy is implemented to facilitate the precise deposition of PVP-based bio-ink at desired positions within each printed layer of collagen to manipulate the pore size within each printed region and eventually fabricate 3D hierarchical porous collagen-based structures. The 3D hierarchical porous collagen-fibroblast matrices serve as the dermal skin constructs for patterning of EMUs.

Lastly, the feasibility of fabricating pigmented human skin constructs with uniform skin pigmentation (using 3 different skin cells from 3 different skin donors) is demonstrated. The histological analysis of the 3D bioprinted pigmented human skin constructs has revealed the similar morphological appearance to the native skin and the immunochemical analysis has indicated the presence of some important biomarkers in native skin. Although 3D bioprinting is an advanced manufacturing platform, it is critical to note that a holistic approach of combining bioprinting-based strategy with other important strategies such as MMC and co-culture techniques has facilitated the fabrication of 3D pigmented human skin constructs with uniform skin pigmentation.

LIST OF PUBLICATIONS

Published Journal Papers

- [1] W. L. Ng, Z. Q. Tan, W. Y. Yeong, & M. W. Naing, "Proof-of-Concept: 3D Bioprinting of Pigmented Human Skin Constructs" *Biofabrication*, **Accepted and Selected for Press Release**
- [2] W. L. Ng, W. Y. Yeong, and M. W. Naing, "Polyvinylpyrrolidone-based bio-ink improves cell viability and homogeneity during drop-on-demand printing," *Materials*, 10,2 (2017) 190.
- [3] W. L. Ng, J. M. Lee, W. Y. Yeong, and M. W. Naing, "Microvalve-based bioprinting – process, bio-inks and applications," *Biomaterials Science*, 5,4 (2017) 632-647.
- [4] W. L. Ng, S. Wang, W. Y. Yeong, and M. W. Naing, "Skin Bioprinting: Impending Reality or Fantasy?," *Trends in Biotechnology*, vol. 34 pp. 689 - 699, 2016. – **Hot paper in Web of Science (top 0.1% of papers in the academic field of Biology & Biochemistry published in past 2 years)**
- [5] W. L. Ng, W. Y. Yeong, and M. W. Naing, "Polyelectrolyte gelatin-chitosan hydrogel optimized for 3D bioprinting in skin tissue engineering," *International Journal of Bioprinting*, vol. 2 pp. 53-62, 2016.
- [6] W. L. Ng, W. Y. Yeong, and M. W. Naing, "Cellular Approaches to Tissue-Engineering of Skin: A Review," *J Tissue Sci Eng*, vol. 6, pp. 1-9, 2015.

Submitted Manuscripts

- [1] W. L. Ng, M. H. Goh, W. Y. Yeong, & M. W. Naing, "Applying Macromolecular Crowding to 3D Bioprinting: Fabrication of 3D Hierarchical Porous Collagen-based Hydrogel Constructs" – Minor revision (Biomaterials Science)

Conference Papers

- [1] W. L. Ng, W. Y. Yeong, & MN. W. Naing, "Microvalve bioprinting of cellular droplets with high resolution and consistency," *In Proceedings of the 2nd International Conference on Progress in Additive Manufacturing*, 2016.

[2] W. L. Ng, W. Y. Yeong, and M. W. Naing, "Development of Polyelectrolyte Chitosan-gelatin Hydrogels for Skin Bioprinting," *Procedia CIRP*, vol. 49, pp. 105-112, 2016.

[3] W. L. Ng, Yeong, W. Y. & M. W. Naing, "Potential of Bioprinted Films for Skin Tissue Engineering," *in Proceedings of the 1st International Conference on Progress in Additive Manufacturing*, 2014.

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1. Introduction

1.1. Background

Predictive *in-vitro* toxicology testing is critical to ensure the safety of marketed products and the global market for *in-vitro* toxicological testing market has been estimated to be around USD 13 billion in year 2016 and it is projected to increase to USD 27 billion by the end of year 2021 [1]. The conventional toxicology study begins with two-dimensional (2D) cell culture systems, animal models and finally to clinical human trials. The 2D cell culture systems are usually non-predictive and elicit poor *in-vivo* responses as they do not recapitulate the complex organization of native 3D tissues [2]. The use of laboratory animals is highly debatable due to the poor accuracy and reliability [3]. Despite the poor accuracy of such animal models, inhumane animal-testing approach for new chemical ingredients provides safety data to satisfy conservative regulatory requirements. Typical animal tests for cosmetics include skin and eye irritation tests whereby the topical application of chemicals on the shaved skin or eyes of the animals is performed to elicit any form of health hazards. These tests can cause considerable pain and distress to the animals such as blindness, bleeding skin, convulsion or even death. In most cases, pain relief is not provided and the animals are usually killed at the end of a test. Notably, the European Union (EU) has implemented a ban on animal testing for cosmetics testing in 2013 and this has resulted in a need for replacement models for toxicology testing.

Although intensive studies on the standard 2D cell culture systems have elucidated numerous important conceptual advances, it is critical to identify the stark contrast between cells growing on conventional 2D tissue culture substrates and cells growing in more physiological 3D environments [4]. The significant differences in the 2D- and 3D- environment have led to considerable changes in the cell morphology,

cell differentiation, cell-cell and cell-matrix interactions. As such, the 3D *in-vitro* human tissue models could potentially replace traditional 2D cell culture systems and animal models due to their enhanced cell-cell and cell-matrix interactions in a more relevant physiological 3D environment [5].

3D bioprinting can be utilized to fabricate complex biological constructs comprising of different types of cells, biomaterials and even growth factors. It offers a highly automated manufacturing platform that enables the fabrication of 3D tissue constructs with good repeatability and flexibility. Most importantly, 3D bioprinting can directly fabricate graded macro-scale structures that closely emulate the native extracellular matrix (ECM) and also precisely deposit specific types of cells at pre-defined position to facilitate critical cell-cell and cell-matrix interactions. Furthermore, micro-scale structures can also be integrated to offer important biomechanical cues at microscale level to facilitate and improve cellular attachment and proliferation. Therefore, 3D bioprinting offers the ability to construct highly-complex designs that comprising of micro- and macro-scale features, thus facilitating the fabrication of skin constructs that fulfil the numerous criteria of a natural skin cell niche.

Most of the prior bioprinting studies focus mainly on the use of keratinocytes and fibroblasts to fabricate 3D skin constructs. The melanocyte, a critical component in the epidermal melanin units [6], is critical for investigation of skin pigmentation in an *in-vitro* 3D physiological tissue construct. Furthermore, the incorporation of melanocytes to the current 3D bioprinted skin constructs would enable the development of pigmented skin constructs for potential cosmetics and toxicology testing. A prior study has demonstrated that the transplantation of skin constructs consisting of melanocytes from Chinese donors (pale pigmentation) on the nude mice resulted in the formation of black-pigmented skin (hyper-pigmentation) [7]. Another study demonstrated the conventional manual-casting of pigmented skin models by using an optimized co-culture medium (use of pro-pigmenting agents and growth

factors) [8]. Although the use of pro-pigmenting agents induced skin pigmentation, uneven skin pigmentation was observed [8].

The development of 3D pigmented human skin constructs presents an alternative approach to investigate the intricate role of various cell-cell, cell-matrix and epithelial-mesenchymal interactions in the modulation of skin pigmentation. This work demonstrated the feasibility of fabricating 3D pigmented human skin constructs using a holistic approach of combining bioprinting techniques and other critical strategies such as the macromolecular crowding (MMC) and co-culture techniques to facilitate the fabrication of 3D pigmented human skin constructs with uniform skin pigmentation.

1.2. Objectives

The project aims to design and develop a methodology for fabrication of 3D pigmented human skin constructs using advanced manufacturing and tissue maturation process

1.3. Scope

The scope of this project includes:

- (i) Developing suitable bio-inks for improved cell viability and homogeneity
- (ii) Engineering the complex 3D microenvironment in collagen-fibroblast matrices
- (iii) Proof-of-concept for bioprinting of 3D pigmented human skin constructs

1.4. Organization of the Thesis

The thesis is organized as follows:

Chapter 1 gives an introduction of the field of *in-vitro* toxicology testing and the motivation to fabricate 3D pigmented human skin constructs using bioprinting approach.

Chapter 2 consists of literature review that presents the skin anatomy, discusses the current progress and limitations of tissue-engineered skin constructs, highlights the need for alternative models for *in-vitro* toxicology testing, analyzes the challenges in fabrication of pigmented skin constructs and lastly presents a comprehensive analysis of the various 3D bioprinting techniques.

Chapter 3 presents the modeling for drop-on-demand bioprinting of bio-inks and investigates the influence of bio-ink properties on cell viability and homogeneity.

Chapter 4 investigates the influence of MMC on collagen fibrillogenesis, cell-matrix remodeling and cellular behavior, proposes an optimal design for improved skin dermal constructs, presents a novel bioprinting strategy to fabricate hierarchical porous collagen-based hydrogel, analyzes the microstructure of 3D printed collagen-based constructs and determines the biocompatibility of this printing strategy.

Chapter 5 presents the proof-of-concept for bioprinting 3D pigmented human skin constructs using different strategies, presents the characterization of the 3D pigmented skin constructs in term of histological and immunochemical analysis.

Finally, the conclusion of the project as well as recommendations for future work is presented in *Chapter 6*.

2. Literature Review

2.1. Skin anatomy and physiology

2.1.1. Structure and function of native skin

The human skin (which measures ~ 2.5 mm in thickness) has a distinctive feature comprising of natural compartmentalization of different type of skin cells (keratinocytes, melanocytes and fibroblasts) that are positioned specifically next to each other in a highly-ordered manner (Fig 2-1) [9]. This unique positioning of different skin cells is critical for important cell-cell interactions that facilitate paracrine and autocrine signaling [10].

Fig 2-1. Schematic drawing of 3D human skin with epidermal and dermal regions [11].

The epidermal region (which measures about 0.2 mm in thickness) contains melanocytes which are surrounded by neighboring keratinocytes and these keratinocytes would undergo a sequential differentiation process to form a multi-layered keratinocyte region across the thickness of epidermal region (highly differentiated keratinocytes furthest away from the basement membrane) [12]. The secretion and storage of extracellular lipids in the stratified layers of keratinocytes provide the important skin barrier function [13].

The main role of melanocytes is to synthesize melanin, which determines the skin pigmentation and offers protection against the detrimental ultraviolet radiation (UV-R). The melanin granules are first produced and deposited within melanosomes, which are transported to the neighboring keratinocytes through the extended dendrites of the melanocytes. The ratio of keratinocytes to melanocytes in the human skin is determined to be around 20:1; a study demonstrated that a minimum density of melanocytes (1.0×10^4 cells/cm²) was necessary to completely restore the skin pigmentation [14]. The presence of collagen VII is critical for the attachment and positional orientation of melanocytes at the basement membrane region [15].

The dermal skin region mainly consists of ECM with a low number of fibroblast cells (typically $0.2 - 2.0 \times 10^5$ cells/cm³) [16], and this dermal skin region can be classified as an upper 'papillary' and a lower 'reticular' dermal region. The papillary dermal region consists of densely-packed collagen fiber bundles while the reticular dermal region consists of highly porous and thicker fiber. The fibroblasts in the dermal skin region secrete important proteins such as collagen, fibronectin, glycosaminoglycans (GAGs) and growth factors.

2.1.2. Keratinocytes

The keratinocytes in the epidermal region proliferate and differentiate to form stratified keratinocyte layers that serve as an impermeable barrier to pathogens. The epidermal region measures about 0.2mm in thickness and the keratinocytes undergo a sequential differentiation process to form 4 unique regions within the epidermis as depicted in Fig 2-1 (namely *stratum corneum*, *stratum granulosum*, *stratum spinosum* and *stratum basale*). The outermost *stratum corneum* is a cornified layer of terminally-differentiated keratinocytes (corneocytes). The next inner layer (*stratum granulosum*) consists of non-dividing flattened keratinocytes. The following layer (*stratum spinosum*) consists of keratinocytes with limited capacity for cell division (early stage of differentiation). Lastly, the basal layer of the epidermal region (*stratum basale*) consists of the proliferative keratinocytes and keratinocyte stem cells. Every keratinocytes in the basal layer undergo a maturation process and differentiate into corneocytes, that form “bricks-and-mortar” arrays with the secreted extracellular lipid lamellae [17].

The change in calcium concentration in the culture medium can alter the proliferation/differentiation of keratinocytes [18], whereby a higher calcium concentration increases keratinocyte differentiation. Another study reported the use of both vitamin D and calcium led to the activation of both phospholipase C (PLC) and protein kinase C (PKC) signaling pathways that are important in keratinocyte differentiation [19]. The proliferation and differentiation of keratinocytes can be manipulated by numerous biological factors; different external stimuli such as calcium concentration, serum concentration and incubation temperature are shown to induce differentiation of keratinocytes [20]. One of the important mechanisms that determine the keratinocyte’s response to external stimuli is the change in protein phosphorylation; this process is characterized by the ligand binding at the receptors to initiate numerous transduction pathways in the keratinocytes. The signal transduction pathways comprise protein tyrosine kinases (PTK), protein kinase A (PKA), protein kinase C (PKC), mitogen-activated protein kinase (MAPK), casein kinase II, phospholipases and cytokine receptor superfamily

[21]. The keratinocyte differentiation process is activated by the tyrosine phosphorylation and tyrosine kinase activities; which is dependent on the calcium concentration (Ca^{2+}), 12-O-tetradecanoylphorbol-13-acetate (TPA) and epithelial growth factor receptor (EGFR). The keratinocyte differentiation is also activated by the PKC pathway [22], which is dependent on Ca^{2+} , phorbol ester and diacylglycerol (DAG). The keratinocytes in a culture medium of reduced Ca^{2+} concentration (0.05mM) exhibit a basal-cell like morphology, whereas the keratinocytes in a culture medium of higher Ca^{2+} concentration (0.12mM) leads to terminal differentiation, indicated by the presence of early differentiation markers and terminal differentiation markers [18].

The use of allogeneic keratinocytes is not suitable for the development of a permanent skin substitute as the allogeneic cells are frequently rejected by the host's immune system [23]. Another major issue encountered is the long cultivation period to achieve sufficient amount of autologous keratinocytes for clinical applications. Some proposed strategies include the use of low calcium medium to boost the proliferation rate of keratinocytes [24], the use of sub-confluent keratinocytes on functionalized plasma treated surfaces [25-27] or fibrin glue [28] and chimeric composition of keratinocytes (a mixture of allogeneic and autologous keratinocytes) [29, 30]. A study has demonstrated that the use of allogeneic neonatal foreskin-derived keratinocytes did not lead to immune rejection and these neonatal cells also exhibit high proliferation and differentiation capabilities [31, 32]. The proliferation of keratinocytes within *in-vitro* 3D constructs is highly dependent on the epithelial-mesenchymal interactions [23]; it was shown that the keratinocytes ceased to proliferate after 1 week and differentiated into discontinuous epithelium in the absence of fibroblasts.

The modulation of substrate stiffness can alter the proliferation, migration and differentiation of the keratinocytes; enhanced keratinocyte migration and proliferation is observed on a stiffer substrate whereas keratinocyte differentiation is more favorable on softer substrate [33]. The use of an air-liquid

interface (ALI) culturing approach is also important for the induction and formation of a fully-stratified epidermal region [34, 35]. A holistic understanding of the different mechanisms that regulate the keratinocyte behavior is important for the maturation of a fully functional epidermal region.

2.1.3. Melanocytes

The main role of melanocytes is to synthesize melanin, which determines the skin pigmentation and offers protection against the detrimental ultraviolet radiation (UV-R). The melanin granules are first synthesized and deposited within melanosomes, which are transported to the neighboring keratinocytes through the elongated dendrites of the melanocytes [36]. The skin pigmentation is determined by the number, size and distribution of the melanosomes within the epidermal region. Interestingly, the number of melanocytes within a specific area of skin is similar for all types of human skin despite the differences in skin colour [37]. The ratio of melanocytes to keratinocytes in the human skin is determined to be around 1:20; a study demonstrated that a minimum density of melanocytes (1.0×10^4 cells/cm²) is essential for complete restoration of the skin pigmentation [14]. The presence of a basement membrane (BM) at the epidermal-dermal junction is essential for the positional orientation of the melanocytes [15], the BM comprises important proteins such as collagen type IV, collagen VII, laminins and nidogens. Without the presence of BM for secure attachment, the melanocytes would migrate to the upper keratinocyte layers and undergo spontaneous pigmentation to form dark-brown spots. It has been shown in a previous study that the interactions between the skin cells in the epidermal and dermal regions are necessary for the formation of the basement membrane. The process is mainly altered by diffusible factors without the need for direct keratinocyte-fibroblast interaction [38].

The epidermal melanin units (EMUs) comprise of keratinocytes and melanocytes found in the epidermal region. The autocrine and paracrine signaling of the keratinocyte-melanocyte complex can be activated by numerous external stimuli [39]. The activated melanocytes secrete more proopiomelanocortin (POMC, precursor of melanocyte-stimulating hormone) and its receptor melanocortin 1 receptor (MC1-R), whereas the activated keratinocytes secrete more alpha-melanocyte stimulating hormone (α -MSH),

basic fibroblast growth factor (bFGF), adrenocorticotrophic hormone (ACTH), nerve growth factor (NGF) and endothelins.

One of the major difficulties in propagation of cultured melanocytes lies in the poor replicative senescence of melanocytes [40]. A recent study demonstrated that the secretion of important growth factors from the keratinocytes improve the proliferation, migration and differentiation of the melanocytes [41]. Intensive research has been performed over the years to convert stem cells into functional melanocytes [42-45] and provide additional sources of melanocytes for tissue engineering applications. There is only limited success on fabrication of 3D pigmented skin constructs [46]; the fabricated 3D pigmented skin constructs did not show matching skin pigmentation as the donor melanocytes [7].

2.1.3. Fibroblasts

The fibroblasts in the dermal region secrete important proteins such as collagen, growth factors, glycosaminoglycans (GAGs), and fibronectin into the surrounding ECM. The skin dermal region comprise mainly ECM with a low number of fibroblasts [16], it can be further classified into an upper 'papillary' and a lower 'reticular' dermal region. The upper papillary dermal region consists of densely-packed and thin collagen fibers, whereas the lower reticular dermal region consists of loosely-packed and thicker fiber bundles as depicted in Fig 2-2. The dermal fibroblasts have distinct lineages and play important role in regulating the dermal architecture; the upper papillary and lower reticular fibroblasts can be classified as superficial and deep fibroblasts respectively [47]. A recent study has demonstrated that the superficial dermal fibroblasts within 3D skin constructs minimized hypertrophic scarring [48] and accelerated the formation of basement membrane and epidermal barrier [49].

Fig 2-2. Schematic drawing of the dermal skin region with varying 3D microstructures

In a 2D fibroblast culture system, there is faster cell migration on softer substrates (95Pa) and higher proliferation rate on the stiffer substrates (4270Pa) [50]. Interestingly, a stark difference in cellular response to substrate stiffness was observed in 2D and 3D systems [51]. The fibroblasts proliferated faster on stiffer 2D surface, whereas a stiffer 3D microenvironment resulted in slower fibroblast proliferation. The fibroblast proliferation rate can also be influenced by other factors such as the

passage number, donor's age and fibroblast phenotype [52]. Another study has reported the use of vitamin C and anti-oxidants to increase migration and proliferation of fibroblasts [52].

Unlike the allogeneic keratinocytes, the use of allogeneic fibroblasts seldom resulted in immune rejection and they can be used for long-term grafting for up to 2 months [23, 53-57]. The allogeneic neonatal fibroblast is an attractive cell source; the neonatal fibroblasts are highly responsive to mitogens [58] and they can be used for long-term expansion [59]. Although the allogeneic fibroblasts are not rejected by the immune system, they are gradually replaced by the infiltrated autologous fibroblasts over time [19]. The use of autologous fibroblasts was shown to reduce scar formation when compared to allogeneic fibroblasts, the results indicated that the use of autologous fibroblasts is more favorable for permanent engraftment [60, 61].

The skin homeostasis is dependent on the epithelial-mesenchymal interactions. The keratinocytes secrete important protein such as interleukin-1 (IL-1), which stimulates the fibroblasts to synthesize and secrete keratinocyte growth factor (KGF), fibroblast growth factor (FGF) and interleukin-6 (IL-6) [62]. These fibroblast-derived growth factors and cytokines in turn help to regulate the proliferation and differentiation of keratinocytes [63]. This indicates the importance of epithelial-mesenchymal interactions and the existence of a double paracrine signaling. Similarly, the fibroblasts play an important role in regulation of melanocyte behavior [64]. Another study reported the secretion of Dickkopf 1 (DKK1) by fibroblasts inhibited the melanocyte proliferation and melanin synthesis [65]. This explains why the native dermal region consists of mainly ECM proteins with a relatively low number of fibroblasts.

2.2. Current progress and limitations of tissue-engineered skin constructs

Over the last four decades, several TE skin constructs have been utilized as therapeutic clinical products for wound management and have also served as alternative *in-vitro* models for toxicology testing. The skin constructs can be categorized into epidermal, dermal and epidermal-dermal (composite) TE constructs and they are presented in Tables 2-1 to 2-3. In the following sections, the different types of skin constructs and their respective functions are discussed and presented.

2.2.1. Epidermal skin constructs

Epidermal skin constructs containing autologous keratinocytes are often cultivated on top of irradiated murine fibroblast feeder layer. These autologous keratinocytes from patient's skin biopsy are usually cultivated and expanded in laboratories over a period of approximately 4 – 5 weeks to obtain fully stratified keratinocyte cell sheets, which are also known as cultured epithelial autografts (CEAs).

Over the last four decades, confluent CEAs have been utilized for treatment of extensive burns and temporary wound dressings are required due to the lengthy cultivation period for CEAs. Furthermore, these CEAs (which typically range between 2 to 8 cell layers thick) do not result in satisfactory healing outcomes [66] and meticulous handling of the fragile cell sheets is required. The use of synthetic carrier templates such as petrolatum gauze backings and silicone membranes can help to provide mechanical support to the fragile cell sheets. Intriguingly, an acid-functionalized silicone membrane was reported to facilitate attachment, proliferation and easy transfer of keratinocyte cell sheets [67]. However, these carrier templates are non-biodegradable and as such it is necessary to subsequently remove them from the wound site. The use of natural biomaterials such as fibrin [68, 69] and hyaluronic acid [70] as delivery system for cultured keratinocytes provides a suitable microenvironment for migration, proliferation and differentiation of keratinocytes and also improves graft adherence. Furthermore, they

can undergo enzymatic degradation *in-vivo* which eliminates the hassle for subsequent removal from the wound sites.

The application of CEA only provides a thin sheet of epidermal cells with missing dermal component and hence resulted in poor take rates as there was no dermis or basement membrane to secure the CEAs to the underlying tissue [71]. As such, collective efforts to improve the clinical outcomes led to the development of dermal skin constructs.

2.2.2. Dermal skin constructs

The biomaterials in dermal skin constructs not only aid in wound bed preparation, but also provide temporary scaffolds for cell attachment and proliferation [72]. Fibroblasts found in the dermis layer of human skin produce collagen, growth factors, glycosaminoglycans (GAGs), and fibronectin to initiate wound healing. Generally, a two-step process is required for the reconstruction of deep wounds (as shown in Fig 2-3). The dermal skin construct is first placed over the wound site for wound bed preparation, followed by the application of an epidermal layer over a well-vascularized dermal layer.

Fig 2-3. Schematic drawing of applications of dermal skin constructs for wound healing.

These dermal skin constructs can be further classified into cell-seeded scaffolds [73] or acellular scaffolds [74-77]. Most of these commercially available dermal skin constructs are acellular scaffolds, which mainly function as temporary scaffolds for cellular infiltration and attachment. This could be due to lower manufacturing costs and straightforward logistics and storage [78]. It was demonstrated that

acellular dermal constructs could be repopulated by autologous fibroblasts *in-vivo* from the underlying wound bed from day 3 onwards [79]. This process is known as “infiltration” and it is responsible for producing a new dermal region which gradually replaces the acellular scaffold as it biodegrades. In contrast, the incorporation of allogeneic human neonatal fibroblasts within the dermal skin substitutes only serves as a temporary source of growth factors and ECM proteins, as these allogeneic fibroblasts would be removed by the host’s immune system within 2 – 3 weeks after implantation [23].

2.2.3. Composite skin substitutes

Epidermal-dermal (composite) skin substitutes comprising both epidermal and dermal layers are currently the most sophisticated tissue-engineered skin product that closely resembles the structure of native human skin. The composite skin substitutes are approximately 0.75 mm in thickness [80] and the presence of both keratinocytes and fibroblasts within the composite skin substitutes leads to the production of a variety of growth factors and cytokines which expedite wound healing [81-83], highlighting the importance of epithelial-mesenchymal interactions. These epidermal-dermal skin substitutes have been utilized for treatment of chronic wounds and ulcers and higher incidences of wound closure are reported [84].

Delayed vascularization in thick tissue-engineered skin constructs (> 0.4mm) remains a critical bottleneck in skin TE [85]. It was highlighted that poor penetration of blood vessels was observed in thicker tissues [86]. Integration of skin constructs with the host vasculature is vital for efficient diffusion of oxygen, nutrients and waste products, as such most cells reside close to the blood capillaries as oxygen and nutrient diffusion limit is approximately 0.1-0.2mm [87]. Different engineering approaches have been proposed to induce the formation of blood vessels *in vitro* [88]. A combination of cell-based [89], biomaterial-based [90, 91] and micro-fabrication approaches [92, 93] could possibly alleviate the problem of delayed vascularization. In the cell-based approach, endothelial cell co-cultures, growth

factor-producing cells or stem/progenitor cells were utilized to accelerate the formation of new blood vessels. Studies have also shown that incorporation of biomaterials such as fibrin [90] or hyaluronic acid [91] within the tissue-engineered constructs can help to improve angiogenesis. In the micro-fabrication approaches, miniature channels are fabricated to enhance the oxygen and nutrient diffusion.

Furthermore, the aesthetic and functional outcomes of these skin substitutes remain unsatisfactory [86]. The lack of pigmentation within the skin substitutes has resulted in white patches resembling vitiligo, which may result in a negative impact on the patient's social life. In addition, the lack of UV protection from melanin (skin pigment) has also resulted in skin blistering when exposed to UV radiation. A spray-on cell suspension, ReCell®, which comprises of non-cultured autologous keratinocytes, melanocytes and fibroblasts, was studied for treatment of vitiligo. In one study, re-pigmentation time took approximately 3-5 weeks but clinical results demonstrated good colour match and high extent of re-pigmentation [94]. Conversely, the results from another study conducted over a period of 4 months demonstrated that re-pigmentation of transplanted skin is highly dependent on the patient's age, whereby unsatisfactory results (less than 65%) were obtained for patients above 30 years old [95].

Table 2-1. List of epidermal skin constructs

Brand Name	Product Form	Cell Source	Biomaterial	Role of Biomaterial	Ref
Epicel® (Genzyme Biosurgery)	Cell Sheet	Autologous keratinocytes on irradiated i3T3 fibroblasts	Petrolatum gauze backing	Improves mechanical properties and minimizes moisture loss	[96]
EpiDex® (EuroDerm AG)	Cell Sheet	Autologous outer sheath hair follicle cells	Silicone membrane	Improves mechanical strength	[97]
MySkin® (Regenerys)	Cell Sheet	Subconfluent autologous keratinocytes on irradiated i3T3 fibroblasts	Silicone sheet with acid-functionalized plasma polymer surface	Facilitates easy transfer of keratinocyte cells, improves cell attachment and proliferation	[98]

Table 2-2. List of dermal skin constructs

Brand Name	Product Form	Cell Source	Biomaterial	Role of Biomaterial	Ref
Dermagraft® (Organogenesis Inc.)	Cell-seeded scaffold	Allogeneic neonatal fibroblasts	Bio-absorbable polyglactin mesh	Bio-absorbable scaffold for temporary cell attachment and proliferation	[73]
DermaPure™ (Tissue Regenix)	Acellular scaffold	-	Decellularized human dermis	Receptive matrix that integrate into the host tissue	-
Hyalomatrix® PA (Anika Therapeutics Inc)	Acellular scaffold	-	Bioresorbable dermal substitute made up of HYAFF® (hyaluronic acid ester)	Bioresorbable scaffold for temporary cell attachment and proliferation, prevents bacterial infection	[74]
KaroDerm® (KaroCell Tissue Engineering AB)	Acellular scaffold	-	Human acellular dermis	Biodegradable scaffold for cellular infiltration	-
Matriderm® (MedSkin Solutions)	Acellular scaffold	-	Bovine dermis collagen coated with α -elastin hydrolysate	Biodegradable scaffold for cellular infiltration and capillary growth	[75]
OASIS® Wound Matrix (Cook Biotech Inc.)	Acellular scaffold	-	Collagen matrix from lyophilized porcine small intestinal submucosa	Biodegradable scaffold that accommodates remodeling of host tissue	[76]
Pelnac™ (Gunze Ltd)	Acellular scaffold	-	Porcine tendon-derived atelocollagen sponge layer and silicone layer	Biodegradable scaffold for cellular infiltration and capillary growth	[77]

Table 2-3. List of composite skin constructs

Brand Name	Product Form	Cell Source	Biomaterial	Role of Biomaterial	Ref
Apligraf® (Organogenesis Inc.)	Cell-seeded scaffold	Allogeneic neonatal foreskin-derived human keratinocytes and fibroblasts	Bovine Collagen Sponge	Biodegradable scaffold that facilitates cell attachment; improves mechanical strength and protects against bacterial infection	[81]
Orcel® (FortiCell Bioscience)	Cell-seeded scaffold	Allogeneic neonatal foreskin-derived human keratinocytes and fibroblasts	Bovine Collagen Sponge	Bio-absorbable matrix that facilitates cell migration	[82]
Permaderm (Regenecin Inc.)	Cell-seeded scaffold	Autologous keratinocytes and fibroblasts	Bovine collagen-glycosaminoglycan scaffold	Biodegradable scaffold that facilitates cell attachment and proliferation	[83]

2.3. Development of *in-vitro* skin constructs for toxicology testing

2.3.1. Challenges in *in-vitro* toxicology testing

The conventional toxicology study begins with two-dimensional (2D) cell culture tests (keratinocyte cell layers), the use of animal models and finally clinical human trials. The 2D cell culture models are usually non-predictive and elicit poor *in-vivo* responses as 2D models do not emulate the complex 3D microenvironment of native tissues [2]. Furthermore, the use of animal testing is highly debatable due to its poor accuracy and reliability [3]. However, inhumane animal-testing approach on new chemical ingredients is still necessary to provide safety data that satisfy conservative regulatory requirements. In addition, a complete ban on animal testing for cosmetics testing by the European Union (EU) in 2013 highlights the need for replacement testing models [99]. Hence, there is currently a huge demand for the design and development of *in-vitro* 3D human skin constructs for such cosmetics and toxicology testing. The development of 3D human tissue constructs would provide more accurate and reliable results than the traditional 2D cell culture systems and animal models [5].

2.3.2. Development of tissue-engineered human constructs

The toxicology testing market is a fast-growing market and there is currently a huge demand for the development of improved *in-vitro* 3D human skin constructs that can advance the toxicology testing [100, 101]. Most of the prior studies on the understanding of tissue morphogenesis were based on 2D cell culture studies or on animal models. Although intensive studies on these standard 2D cell culture have elucidated numerous important conceptual advances, it is critical to identify the stark contrast between cells growing on flat 2D substrates and cells growing in more physiological 3D microenvironment [4]. The significant differences in the 2D- and 3D- environment led to considerable changes in the cell morphology, cell differentiation, cell-cell and cell-matrix interactions. As such, the 3D *in-vitro* human tissue models could potentially replace simple 2D cell cultures and animal models due to their enhanced cell-cell and cell-matrix interactions in a more relevant physiological 3D environment [5].

The development of TE skin constructs first began 40 years ago [102, 103], when keratinocytes were first propagated in the laboratory as 2D cell sheets. Since then, intensive research on cell biology has brought about significant advances in skin tissue engineering [89, 104]. Owing to the rigorous research and valuable knowledge, tissue-engineered skin has been a reality for a long time [86]. An organotypic human skin construct typically comprise stratified layers of keratinocytes and a contracted collagen-fibroblast matrix [13]. The evaluation of such organotypic skin models in term of morphological, biochemical and physiological properties has indicated that the artificial skin constructs have certain degree of resemblance to the native skin tissue; the presence of well-stratified keratinocyte layers (indicated by a cornified envelope and presence of numerous tonofilaments and desmosomes).

2.3.3. Limitations of existing 3D tissue-engineered human skin constructs

Human skin is a complex organ with well-defined spatial structures that consist of multiple types of cells. At present, none of the tissue-engineered skin constructs can fully replicate native skin in terms of morphological, biochemical and physiological properties. Overall, relatively simple skin constructs consisting of keratinocytes and fibroblasts are already commercially available. Despite the advances in skin tissue engineering, some of the major bottlenecks that remain to be solved include the lack of skin pigmentation and missing hair follicles. The hair follicle is an interesting feature on the skin and the self-regeneration of new hair follicles through the hair cycle suggested the presence of intrinsic stem cells. The highly proliferative capacity and multi-potency of the hair follicle stem cells is gaining huge attention due to its ability to rejuvenate the different skin appendages [105]. Numerous attempts to induce *de novo* hair follicle growth in human have failed due to the loss of key inductive properties in dermal papilla cells. A recent study has demonstrated that their hair-inducing properties can be partially restored in a 3D environment [106]. Although the ability to closely emulate this functional niche could potentially initiate hair neogenesis between the dermal papillae and epidermal cells, the restoration of hair follicles in TE skin remains a highly-complex genetic problem.

Lack of skin pigmentation

Skin pigmentation is closely associated with the transfer of melanin from a melanocyte to its surrounding keratinocytes and visible pigmentation is primarily found within the keratinocytes [107]. The incorporation of melanocytes to develop 3D pigmented human skin constructs would provide a highly relevant tool for *in-vitro* cosmetics testing and fundamental study of critical epithelial-mesenchymal interactions in the regulation of skin pigmentation. The key challenges in development of 3D pigmented human skin constructs include the development of optimal co-culture medium to support all three types of skin cells (keratinocytes, melanocytes and fibroblasts), the patterning of functional

epidermal melanin units (EMUs) and the restoration of a basement membrane in the 3D pigmented human skin constructs. The ratio of epidermal melanocytes to surrounding keratinocytes in native skin is approximately 1:20 and a minimum density of 1.0×10^4 melanocytes/cm² is required to completely restore the skin pigmentation [14]. The ability to pattern keratinocytes and melanocytes in a similar fashion (to emulate EMUs) is important for melanin synthesis and transfer. Furthermore, the presence of basement membrane (BM) components at the epidermal-dermal junction is necessary for secure attachment of melanocytes [15] and melanin synthesis through regulation of tyrosine uptake [108].

2.4. Epidermal melanin units: building blocks for skin pigmentation

The epidermal melanin unit (EMU) is a functional complex unit found within the epidermis that consists of both keratinocytes and melanocytes (Fig 2-4). The melanocytes produce and store the melanin granules within specialized organelles called melanosomes [107]. The skin pigmentation that one sees is primarily due to the melanin granules stored within the keratinocytes. The melanocytes extend their long dendrites that can contact up to 40 keratinocytes for the transfer of melanin within the epidermal melanin units.

Fig 2-4. Epidermal-melanin units at the epidermal-dermal junctions.

The skin colour is determined by the types of melanin produced within the epidermal skin region and the melanin distribution in the native skin. Despite the differences in skin pigmentation, the number of melanocytes in the native skin is similar for all ethnic groups [109]. However, the number of melanocytes is known to vary across the different anatomical sites of the human body [110]. The melanocytes transport melanosomes via extended dendrites to the surrounding keratinocytes [111] and these melanosomes serve as an important barrier to protect DNA from the harmful ultraviolet radiation [112].

2.4.1. Regulation of skin pigmentation

The modulation of skin pigmentation is a highly-complex mechanism and it involves the regulation of more than 125 genes [113]. The presence of important structural and enzymatic proteins is necessary for melanin production [114, 115]. The skin pigmentation is determined by the types and ratio of melanin found in the skin; the three types of melanin are the yellowish-brown pheomelanin, the dark brown eumelanin and the black eumelanin [116].

Three important enzymes are responsible for the melanin synthesis (Fig 2-5). Tyrosinase (TYR) is an enzyme that catalyzes the hydroxylation of tyrosine to dihydroxyphenylalanine (DOPA). The DOPA is then subsequently oxidizes to form DOPAquinone. The DOPAquinone can undergo 2 different chemical pathways to form pheomelanins or DOPACHrome depending on the presence or absence of cysteine or glutathione [117, 118]. The DOPACHrome is converted into DHI-2-carboxylic acid (DHICA) in the presence of the enzyme DOPACHrome tautomerase (TYRP-2) [119]. The DHICA melanins and DHI melanins are converted into indole-5,6-quinone-carboxylic acid and indole-5,6-quinone respectively and combine to form eumelanin.

Fig 2-5.Process of melanogenesis within epidermal melanosomes [120]

The tyrosinase-related protein 1 (TYRP1) is an important enzyme for the stabilization of tyrosinase for melanin production [121] and the presence of DOPachrome tautomerase (TYRP-2) also play an important role in regulating melanocyte growth and morphology [122]. The mutations in these enzymes could result in significant changes in the quantity and quality of synthesized melanin. Tyrosinase is an important enzyme in the melanogenesis process; mutations can severely affect tyrosinase function and potentially result in albinism [123]. Proper regulation of enzymes and pH within the melanosomes by the proteins is critical for the melanin production. In following sections, different factors that influence the skin pigmentation are presented.

UV radiation

The change in skin pigmentation due to external stimuli can be classified as facultative skin pigmentation [37]. Among the many factors that modulate skin pigmentation, UV radiation plays the most critical role in influencing skin pigmentation. UV radiation can be classified as UVA (320 – 400 nm), UVB (290 – 320 nm) and UVC (< 320 nm). UVA rays are the dominant UV radiation (95% of total radiation) and UVA radiation leads to instantaneous darkening of skin pigmentation within minutes [124]. The skin tanning effect is not induced by an increase in melanin synthesis but it is mainly due to the re-distribution of existing melanosomes within the epidermal region and oxidation of existing melanin. The effect of delayed tanning usually occurs several days after prolonged UV exposure. The prolonged UV exposure leads to an increase in MITF (the master transcriptional regulator of pigmentation) expression and other melanogenic proteins [37]. Furthermore, an increased expression of PAR2 by the keratinocytes also enhances the melanosome uptake and distribution in the epidermal region [125].

Melanocytes, keratinocytes and fibroblasts react to UV radiation by releasing a wide variety of melanogenic factors. Both melanocytes and keratinocytes demonstrate elevated expression of proopiomelanocortin (POMC) and the melanocortin precursors. The UV radiation also induces the keratinocytes to secrete more endothelin-1 (ET-1) which enhances melanocyte functions [126]. Other important protein such as interleukin-1 (IL-1) stimulates the autocrine secretion important for melanogenesis [127]. The activation of p53 pathway in keratinocytes also induces elevated expression of the POMC gene which results in enhanced melanin synthesis in melanocytes [128]. Similarly, the exposure to UV radiation induces increased secretion of fibroblast-derived growth factors which play critical role in melanogenesis [129].

Physical topography

The cells within the native tissue reside in a highly-complex 3D microenvironment; little information is known about how the physical signals from the surrounding 3D microenvironment influence the cells. In one study, healthy human melanocytes were seeded onto poly-dimethylsiloxane (PDMS) substrates patterned with stripes to investigate the influence of physical surface topography on cellular behavior (2D cell-matrix system) [130]. The influence of the mechano-physical signals was studied in a systematic manner by increasing the height of the microstructures. The melanocytes are responsive to changes in surface topographical features and the study has showed that melanocyte interaction with microstripes improved melanin production significantly.

Biochemical compounds

The regulation of skin pigmentation is a complex mechanism; numerous biochemical compounds have been applied to regulate the skin pigmentation via different mechanisms. The different approaches include the inhibition of tyrosinase enzyme and its related melanogenic catalytic pathways, regulation of melanogenesis and down-regulation of the melanosome transfer within the epidermal melanin units. Numerous depigmenting agents have been developed to manage skin hyperpigmentation; extensive review on the different depigmenting agents has been covered elsewhere [120].

2.4.2. Challenges in fabrication of 3D pigmented skin constructs

Most of the prior studies focus mainly on the use of keratinocytes and fibroblasts to fabricate 3D human skin constructs. The melanocyte, a critical component in the epidermal melanin units [6], is critical for investigation of skin pigmentation in an *in-vitro* 3D physiological model. Furthermore, the incorporation of melanocytes to the current 3D bioprinted skin constructs would enable the development of 3D pigmented human skin constructs for potential cosmetics and toxicology testing. The fabricated pigmented skin substitutes that were transplanted on the nude rats displayed darker pigmentation that is significantly different from the constitutive pigmentation of the donor skin [7]. The excessive soluble factors from the murine models might have led to the increase in skin pigmentation [64].

In a recent study, the use of pro-pigmenting agents helped to induce the melanogenesis process and achieve pigmentation in the 3D human skin constructs [8]. However, the issue of uneven skin pigmentation still persists. The important considerations for development of 3D pigmented human skin constructs include the formulation of optimal co-culture medium to support all three types of skin cells (keratinocytes, melanocytes and fibroblasts), the patterning of keratinocytes and melanocytes at the epidermal region to emulate the functional epidermal melanin units (EMUs) and the restoration of basement membrane proteins at the epidermal-dermal junction of 3D bioprinted human skin constructs.

2.5. Fabrication techniques

After identifying the critical characteristics that are crucial towards the design and fabrication of an ideal biomimetic 3D pigmented human skin construct, the next step is to utilize a suitable fabrication technique to create the envisaged construct. In the following sections, the working principles, advantages and limitations of different fabrication approaches are discussed and evaluated.

2.5.1. Conventional fabrication approaches

Freeze drying

Freeze drying process enables fabrication of 3-D porous scaffolds via the sublimation of water crystals and these pore sizes can be manipulated by different parameters such as freezing rate, ionic concentration, temperature and pH [131] (Fig 2-6). The polymer is first poured into a mold and froze overnight between -20°C to -40°C , followed by lyophilization for several days depending on size of the scaffold [132-135]. For indirect fabrication of freeze-dried scaffolds, computer-aided model of a pre-defined sacrificial mold is first created and then fabricated using a phase-change inkjet printer. The polymer is then cast into a pre-defined mold with channels and froze overnight, followed by the lyophilization. The sacrificial mold is removed by dissolving it in ethanol to obtain a porous construct with pre-defined interconnected channels [136, 137] that enhance the mass transfer of oxygen and nutrients to the interior of the scaffold. Although highly porous freeze-dried scaffolds with interconnected pores could be obtained, it is challenging to achieve precise control over the pore sizes, distribution and interconnectivity of pores within the scaffolds.

Electrospinning

Electrospinning is a relatively simple technique that uses electrostatic forces to fabricate porous scaffolds with micro- to nano-scale polymer fibers [138-141] (Fig 2-6). Electrospinning can be used for the development of biomimetic scaffolds that closely resemble the structure and biological function of native ECM with nano-scale structures. A comprehensive review on electrospinning has been covered elsewhere [142], highlighting the influence of different processing parameters on the fiber formation and structure. The nano-fibrous electrospun scaffolds are structurally similar to the collagen multi-fibril network (50-250nm) present in the native ECM [143] and such nano-scale fiber diameter plays an imperative role in regulating cellular behavior. It has been demonstrated that cell adhesion and proliferation are more effective within nanofiber-based scaffolds as compared to microfiber-based scaffolds [144]. Nevertheless, some limitations of electrospinning include poor cell penetration and weak mechanical property. The formation of a well-stratified epidermal layer requires an inter-fiber distance of at least 10 μ m for improved cell penetration [145]. Furthermore, the weak mechanical property of such nanofiber-based scaffolds can be enhanced by several approaches such as annealing at elevated temperatures, the use of rotating collector to achieve higher degree of fiber alignment and cross-linking of fibers by irradiation or chemical means [146].

Fig 2-6. SEM images of (left) freeze-dried scaffold with micro-pores (scale bar: 50 μ m) and (right) nano-fibrous electrospun scaffold (scale bar: 10 μ m) (unpublished data)

Decellularization

Recently, there is a growing interest in the decellularization of organs/tissues to obtain biomimetic scaffolds for tissue engineering applications. Decellularization is the removal of cells from an organ/tissue to preserve the structural composition of the native ECM and it is an attractive technique of harvesting native extracellular matrices for skin tissue engineering applications. A combination of physical, enzymatic and chemical decellularization techniques are typically utilized [147]. The decellularized matrices serve as instructive 3D biological scaffolds for the reconstruction of functional tissues via infiltration of autologous cells.

The major advantage of decellularization process is the preservation of intricate ECM structures and the presence of an intact vascular network which can anastomose to host vessels upon transplantation [148]. Although incomplete removal of cellular remnants may induce pro-inflammatory responses, a combination of qualitative and quantitative techniques could be employed to mitigate such detrimental reactions [148]. Despite its attractiveness in providing biomimetic scaffolds with intricate internal micro- and nano-structures, the key drawback of this technique links back to the problem of limited availability of skin donor sites and the use of xenogeneic skin could lead to possible risks of immune complications [147].

Evaluation of conventional fabrication approach

Over the last three decades, top-down fabrication techniques such as freeze drying [132-135] and electrospinning [138-141] for skin TE have been reported and they have led to scientific progress and partial clinical successes for wound treatments. Nevertheless, the engineering of a complex

heterogeneous construct remains challenging due to the intrinsic limitations of traditional top-down fabrication approach. There is limited cell penetration depth and it remains difficult to manipulate the deposition of different types of skin cells at high degree of specificity within the 3D scaffolds [149]. As such, the top-down fabrication strategy is not a suitable approach for fabrication of highly complex 3D constructs with functional organization.

2.5.2. 3D Bioprinting approaches

Bioprinting-based approach

Bioprinting provides a fully automated and advanced platform that facilitates simultaneous and highly specific deposition of multiple types of skin cells and biomaterials, which is lacking in conventional skin tissue engineering approaches. The goal of this section is to provide a realistic overview of the current skin bioprinting works. An in-depth analysis of current skin bioprinting works and a detailed analysis of the cellular and matrix components of native human skin are presented. This section will also highlight the current limitations and achievements specifically in skin bioprinting, followed by design considerations and a future outlook on skin bioprinting. The potential of bioprinting with converging opportunities in biology, material and computational design will eventually facilitate the fabrication of improved tissue-engineered skin constructs, making bioprinting of pigmented skin an impending reality.

3D bioprinting is an emerging field that can be utilized to fabricate complex biological constructs comprising of different types of cells, biomaterials and even growth factors. It offers a highly automated manufacturing platform that enables the layer-by-layer manufacturing of 3D tissue constructs with high degree of flexibility and repeatability (Fig 2-7). Most importantly, 3D bioprinting has the potential to directly fabricate graded macroscale structures that closely resemble the native extracellular matrix (ECM) and also precisely deposit specific types of cells at pre-defined position to facilitate critical cell-cell and cell-matrix interactions [11]. Furthermore, micro-scale structures such as ridges and modulated surfaces can be incorporated to provide mechanical and biochemical cues at microscale level to facilitate and improve cellular attachment and proliferation. Hence, bioprinting offers concurrent engineering design that spans across micro- and macro-scales, thus enabling the fabrication of skin constructs that can better satisfy the various requirements of a natural niche for skin cells. The combination of a programmed printing process and tissue engineering enables large-scale production of

tissue-engineered skin constructs, while offering flexibility in design and fabrication of customized skin construct. In general, a higher printing resolution would result in a longer manufacturing time for a given processing speed. Hence, it is important to maintain a balance between printing resolution and manufacturing scale. Bioprinting approaches such as laser-based [150-152] and microvalve-based [92, 153-155] printing techniques are used to manufacture multi-layered 3D skin constructs with high cell viability and proliferation.

Fig 2-7. Schematic drawing for skin bioprinting process; the different types of skin cells are harvested and printed to obtain the 3D bioprinted human skin constructs [11].

Microvalve-based bioprinting

A typical microvalve-based bioprinting setup consists of an array of microvalves and a three-axis movable robotic stage. Each microvalve is connected to an individual gas regulator that provides the pneumatic pressure and the valve opening time ($\sim\mu\text{s}$) is controlled by a pulse generator as shown in Fig 2-8. Microvalve-based bioprinting could be utilized for direct deposition of cell-laden hydrogels or the simultaneous deposition of cell droplets and matrix materials [156]. The material deposition process is dependent on the nozzle diameter, material viscosity, pneumatic pressure and the valve opening time [155]. It facilitates the controlled deposition of materials in x-y direction as directed in the pre-defined computer-aided design (CAD) file and the deposited layer serves as a foundation for the subsequent layers.

Fig 2-8. Schematic drawing of microvalve-based bioprinting process

Multi-layered collagen constructs containing keratinocytes and fibroblasts were crosslinked using nebulized aqueous sodium bicarbonate on a non-planar surface [155]. The printed cells demonstrated normal proliferation. Subsequently, fluidic channels were fabricated using a sacrificial gelatin template [92]. Significantly higher cell viability was observed in the perfused 3D constructs with fluidic channels (85% viability) as compared to the ones without any channels (60% viability) (Fig 2-10B). Another work demonstrated the ability to emulate native cellular density of different skin cells within bioprinted skin constructs (Fig 2-10B) [154]. Collagen layers were first printed, followed by the deposition of keratinocytes and fibroblasts on top of each specific collagen layers. A time lapse of one minute was

required for crosslinking of each collagen layer prior to printing of subsequent layers and high cell viability (> 94% for both keratinocytes and fibroblasts) was reported. Although the printed construct maintained its overall shape when cultured under submerged culture condition for 7 days, shrinkage of the printed collagen construct was observed when cultured at air-liquid interface (ALI). Furthermore, there was an incomplete stratification of the epidermal layer after 3 weeks of tissue maturation.

Laser-based bioprinting

A typical laser-based bioprinting setup consists of a pulsed laser beam, a focusing system, a 'ribbon' (a donor slide coated with a layer of energy-absorbing layer and cell-encapsulated hydrogel) and a collector slide facing the ribbon as shown in Fig 2-9. High powered energy from a moving pulsed laser beam is first absorbed by the energy-absorbing layer, the local evaporation of the energy-absorbing layer then created a high gas pressure which propelled the cell-encapsulated hydrogel toward the collector slide [152]. The process is repeated to fabricate a multi-layered construct with precise cellular deposition at specific positions.

Fig 2-9. Schematic drawing of laser-based bioprinting process

A recent *in-vitro* study demonstrated the deposition of 20 layers of fibroblasts (mouse NIH-3T3) and subsequent 20 layers of keratinocytes (human HaCaT) embedded in collagen gel onto a sheet of Matrigel® (decellularized dermal matrix) (Fig 2-10B) [151]. Evaluation of the printed skin constructs after 10 days of cultivation showed presence of cadherins and connexin 43 (Cx 43) in the epidermis, which are fundamental for tissue morphogenesis. Furthermore, bioprinted 3D grafts comprising adipose-derived stem cells expressed adipogenic markers resembling those found in native adipose

tissue [157]. In another study, the *in-vivo* transplantation of a printed skin construct in the dorsal skin fold chamber of nude mice demonstrated good graft-take with the surrounding tissue and ingrowth of some blood vessels from the wound bed was observed after 11 days of transplantation [152].

Fig 2-10. (A) Different studies on skin bioprinting are presented. (B) The bioprinting features important for skin bioprinting [17,20]. (C) Comparison of native human skin [67] and bioprinted skin constructs [18] (scale bar: 100 μm).

Evaluation of bioprinted skin constructs

Skin is a complex organ with well-defined spatial structure that consists of multiple types of cells. At present, none of the bioprinted skin constructs can fully replicate native skin in terms of morphological, biochemical and physiological properties (Fig 2-10C). Overall, relatively simple skin constructs consisting of keratinocytes and fibroblasts have been successfully printed using bioprinting techniques [151, 152, 154]. Those skin constructs demonstrated some resemblance to native skin and conferred some skin functions in *in-vivo* studies.

Morphological properties

The ECM and cellular density can be controlled as independent design parameters in bioprinting and the printing process was able to maintain good cell viability [92, 151, 152, 154, 155]. The printed construct [154] comprised an epidermal region with densely-packed keratinocytes and a dermal region with predominantly collagen type I and relatively low fibroblast density ($0.2 - 2 \times 10^5$ fibroblasts/cm³) [158] resembling native skin. The presence of collagen IV and laminin at the epidermal-dermal junction indicated formation of basement membrane [151, 152]. Furthermore, the thicknesses of printed constructs can be customized to match the wound depth [155]. Although the printed constructs were cultured at an air-liquid interface to promote maturation of the epidermal region [159], incomplete epidermal stratification and keratinization of printed constructs were observed [152, 154]. The use of immortalized keratinocyte cells in these studies could have resulted in the gradual loss of cell phenotype and function.

Biochemical properties

The *in-vivo* transplantation of the printed skin construct in nude mice formed thickened layers of epidermal region exhibiting early stage of differentiation and stratification after 11 days [152]. The

expression of cytokeratin 14 (keratin intermediate filament) in the whole epidermis and the presence of Ki67 (proliferation marker) mainly in the suprabasal layers indicated early differentiation of keratinocytes. Furthermore, presence of cadherins between adjacent keratinocytes highlighted crucial cellular interactions that is important for epidermal formation and consequently tissue development [152, 154].

Physiological properties

The barrier function of the skin is closely linked to the formation of *stratum corneum* (terminally-differentiated keratinocytes) at the outermost epidermal region [9]. The barrier function of the bioprinted constructs is not yet fully functional due to the absence of completely differentiated epidermal region in the bioprinted constructs [152, 154]. Furthermore, blood vessels from the underlying tissue [152] is important for oxygen and nutrient exchange. The printed skin constructs can be evaluated via different approaches such as histological analysis, immunohistochemistry, gene expression and mechanical testing. Hematoxylin and eosin stain (H &E) staining is generally performed to identify the presence and morphology of different types of skin cells. Specific protein markers expression can be monitored through immunohistochemistry to assess epidermal differentiation, formation of new blood vessels and cell proliferation/migration [160]. The epidermal barrier function of the skin can be evaluated via transepidermal water loss (TEWL).

Evaluation of 3D bioprinting approaches

The bioprinting approach is an automated process that offers flexibility in the design of the construct and facilitates simultaneous deposition of cells and biomaterials to obtain a complex multi-cellular construct. The main roles of human skin are to provide the skin barrier function (prevent entry of microbes and excessive trans-epidermal water loss) and protection against the UV radiation. The

incomplete stratification of the epidermal layers within the matured bioprinted constructs would result a breach in the skin barrier function; incorporation of biological cues could provide guided tissue maturation into a complete stratified epidermal layer. In addition, absence of melanocytes in the bioprinted skin construct would result in non-pigmented skin constructs and the reconstruction of a basement membrane layer within the tissue-engineered skin construct is required for the secure attachment of these melanocytes. Without this basement membrane layer, melanocytes would migrate to the superficial keratinocyte layers and undergo spontaneous pigmentation to form dark brown spots. It would be desirable to leverage the strengths of different biomaterials for fabrication of 3D improved skin constructs. The preliminary bioprinting results are promising and more intensive work could be done to improve on the bioprinted skin constructs. Despite being in the stage of infancy, bioprinting approach has promising potential for fabrication of 3D pigmented human skin constructs via patterning of multiple types of cells and biomaterials at pre-defined regions.

2.5.3. Design considerations for 3D bioprinting of pigmented human skin constructs

Development of bio-inks for improved cell viability and homogeneity

A major issue for bioprinting is the low cell viability and poor cell homogeneity during printing process. To date, numerous studies have been conducted to evaluate the effects of printing pressure [161-163], nozzle diameter [161-163] and substrate stiffness [164] on cell viability during the printing process. Furthermore, another challenging issue faced in most bioprinting systems is cell sedimentation effect, which occurs as a result of gravitational forces and changes the cell homogeneity within the printing cartridge over time. This cell sedimentation effect on bioprinter output was characterized [165], highlighting the issue of inconsistent printing output over time. Some attempts to mitigate the sedimentation effects include the use of ethylene diamine tetra-acetic acid (EDTA) [166] and neutral buoyancy [167]. However, the use of EDTA can be detrimental to cell viability and the direct measurement of cell density poses a huge obstacle due to the lack of sophisticated measuring equipment (most measurements are only limited to indirect means through density centrifugation or optical techniques) [167]. The key limitation that hindered the prevalent use of cell-based bioprinting is due to the poor viability and homogeneity of the printed cells [168] and it is important to evaluate the influence of bio-ink properties on the printed cells.

Cellular requirements

Native skin tissue comprises multiple types of cells with specific biological functions that should be recapitulated in tissue-engineered constructs [9]. Cell sourcing is a critical component of bioprinting (Fig 2-13). The use of autologous cells eliminates potential risk of rejection; primary cells such as keratinocytes, melanocytes and fibroblasts can be isolated from skin biopsy and large-scale industrialized cell culture expansion is necessary for bioprinting applications [169]. The use of stem cells

[170] may provide potential solution to overcome the limitations of current technologies (e.g., the lack of vascular networks, sensory receptors and skin appendages). Using stem cells has been partially effective, but more extensive studies are required to evaluate the potential risks of malignant teratoma formation and long-term adverse effects of the stem cells. Different strategies could be implemented to accelerate the maturation of bioprinted constructs into functional tissues [171]. One of major challenges is the incorporation of additional cell types in the current co-culture system of keratinocytes and fibroblasts; this poses a challenge in striking a balance between proliferation and the differentiation of multiple cell types. Another critical aspect is to create the functional epidermal melanin units via patterning of keratinocytes and melanocytes in an optimal cellular density and ratio. Skin pigmentation is closely associated with the transfer of melanin from a melanocyte to its surrounding keratinocytes and visible pigmentation is primarily found within the keratinocytes [107]. The ratio of epidermal melanocytes to surrounding keratinocytes in native skin is approximately 1:20 and a minimum density of 1.0×10^4 melanocytes/cm² is required to completely restore the skin pigmentation [14]. The ability to pattern keratinocytes and melanocytes in a similar fashion is important for melanin synthesis and transfer. Furthermore, the presence of basement membrane (BM) components at the epidermal-dermal junction is necessary for secure attachment of melanocytes [15] and melanin synthesis through regulation of tyrosine uptake [108].

Functionally-graded constructs

Skin is a functionally-graded organ with anisotropic distribution of both cellular and ECM components [10]. The dermal region is characterized by a relatively higher number of fibroblasts in the upper “papillary” region in comparison to the lower “reticular” region while the epidermal region is characterized by the presence of keratinocytes and melanocytes in an optimal ratio at the epidermal-dermal junction [14]. The ability to pattern different cell types at a desired cellular density is critical in

achieving biological functionally-graded constructs (Fig 2-13). The dermal region comprises an upper “papillary” region characterized by densely-packed thin collagen fibers (small pores) and a lower “reticular” region depicted by loosely-packed thick collagen fibers (big pores) [10]. The ability to design and fabricate such a 3D hierarchical porous collagen-based structure would result in biomimetic dermal skin constructs.

Macromolecular crowding (MMC) is a concept that has been developed to describe the inter- and intra-cellular space;[172] it is a phenomenon that drives the cell biochemistry through its effect on fundamental processes such as protein folding and interactions with nucleic acids. MMC can shift the reaction equilibria and accelerate the rates of numerous chemical reactions such as enzymatic catalysis, receptor-ligand interactions and supramolecular aggregation.[173] Most of the prior works on MMC incorporated macromolecules within the culture media to reduce the disparity between the *in-vivo* and *in-vitro* microenvironment and the commonly-used macromolecules include dextran sulfate, Ficoll 70, Ficoll 400 and Ficoll mixture (different ratio of Ficoll 70 and 400).[174-176] The addition of such macromolecules into the culture medium enhanced the extracellular matrix (ECM) deposition by living cells in 2D cell culture systems. A surrogate marker known as fractional volume occupancy (FVO), which is highly dependent on the hydrodynamic radii (R_H) of the macromolecules, is used to assess the degree of crowdedness within the solution.[177] The physiological range of FVOs have been evaluated by calculating the total FVO of albumin, fibrinogen, globulin in the blood plasma and interstitial fluid, which lies in the range of 9 – 54% FVO.[178] There has been little work performed on the influence of MMC in a 3D cell culture system; recent studies on MMC in 3D cell culture systems have demonstrated that the presence of macromolecules (Ficoll 400, up to 25 mg/mL ~ 8% FVO) can influence the collagen fibrillogenesis process.[179, 180] A recent work demonstrated that polyvinylpyrrolidone (PVP, MW: 360 kDa) macromolecules have been employed at significantly higher FVOs as compared to other reported macromolecules in a 2D cell culture system to enhance extracellular matrix (ECM) deposition and cell

proliferation in a dose-dependent manner [178]. The presence of macromolecules alters the fibrillogenesis process via excluded volume effect (EVE) and the use of a printable macromolecule-based bio-ink could be employed to tune the pore sizes within a 3D-printed collagen-based matrix via DOD bioprinting to create 3D hierarchical porous structures.

Fig 2-11. Important design considerations for skin bioprinting to achieve skin pigmentation [11]

2.6. Research methodology

Earlier in this chapter, the existing limitations of current tissue-engineered skin constructs have been critically reviewed. One of the major limitations of existing tissue-engineered skin constructs is the lack of skin pigmentation. Most of the prior bioprinting studies focus mainly on the use of keratinocytes and fibroblasts to fabricate functional and biomimetic skin constructs. The melanocyte, a critical component in the epidermal melanin units, is critical for investigation of skin pigmentation in an *in-vitro* 3D physiological model. There is only partial success in the fabrication of 3D pigmented skin constructs. A prior study demonstrated that the transplantation of skin equivalents consisting of melanocytes from Chinese donors (pale pigmentation) on the nude mice resulted in the formation of black-pigmented skin [7]. In another recent study, the use of pro-pigmenting agents induced skin pigmentation in the manual-cast 3D pigmented skin models [8]. However, non-homogeneous skin pigmentation is a key problem in the manual-casting approach. A RegenHU Biofactory® is used for fabrication of 3D pigmented human skin constructs. Being a multiple print-heads system, the RegenHU bioprinting system facilitates the deposition of multiple biomaterials and living cells simultaneously. The microvalve-based print-heads are used to precisely deposit the biomaterials and living cells in a drop-on-demand manner.

2.6.1. Developing suitable bio-inks for improved cell viability and homogeneity

In this project, a suitable bio-ink is developed for the deposition of living cells (different types of skin cells) with improved cell viability and homogeneity. The major issues with cell bioprinting include the high shear stress during the printing process and the inconsistent cell output over printing time. Hence, an in-depth understanding of the influence of bio-ink properties (polymer and cell concentration) on the viability and homogeneity of printed cells is important.

2.6.2. Engineering the complex 3D microenvironment in collagen-fibroblast matrices

The 3D microenvironment of the dermal skin region has a highly-complex 3D architecture; the skin dermal region serves as a critical foundation that provides critical signaling cues that regulate the skin homeostasis. An in-depth understanding the influence of complex 3D microenvironment on cellular behavior would be critical for the development of 3D pigmented human skin constructs. Prior studies have demonstrated that the macromolecular crowding (MMC) is an attractive strategy to alter the collagen fibrillogenesis and cellular behavior. Therefore, first part of the study aims to understand the synergistic effect of MMC on the 3D collagen-fibroblast matrices. Investigations are performed to evaluate the influence of MMC on the intricate cell-matrix interactions and cellular behavior in a 3D microenvironment. An optimal skin dermal region will then be fabricated based on the critical information from the study of cell-matrix interactions in a 3D microenvironment. The use of MMC has a synergistic effect on the 3D collagen-fibroblast matrices; it has resulted in a drastic change in the 3D microenvironment and also influenced the cell-matrix remodeling process and cellular behavior. The experimental results have indicated the importance of hierarchical porous structures within the 3D architecture of the skin dermal region. A novel bioprinting strategy is then implemented to fabricate such a biomimetic 3D microenvironment consisting of a hierarchical porous collagen-based matrix using macromolecule-based bio-ink.

2.6.3. Proof-of-concept: bioprinting 3D pigmented human skin constructs

As a proof-of-concept, the 3D pigmented human skin constructs are fabricated via a two-step bioprinting strategy. The studies from the earlier chapters are critical toward the bioprinting of the 3D pigmented skin constructs. The bioprinting strategy involves the patterning of epidermal melanin units (using a suitable bio-ink that improves cell viability and homogeneity) on top of biomimetic 3D bioprinted skin dermal constructs with hierarchical porous structures. The bioprinted 3D skin constructs

are then cultured in an optimal co-culture medium prior to performing in-depth characterization such as histological and immunochemical analysis.

3. Development of Suitable Bio-inks for Improved Cell Viability and Homogeneity

3.1. Background on microvalve-based bioprinting process

A typical microvalve-based bioprinting system comprises a three-axis movable robotic platform and an array of multiple electromechanical microvalve print-heads [181]. Each microvalve print-head is connected to an individual gas regulator that provides the pneumatic pressure (positive pressure) and the valve opening time (minimum of 0.1 ms) is controlled by movement of both plunger and the solenoid coil. The applied voltage pulse induces a magnetic field that opens the nozzle orifice by pulling the plunger up in an ascending motion. The bio-ink is deposited when the pneumatic pressure overcomes the fluid viscosity and surface tension at the opened orifice. The material deposition process is dependent on the nozzle diameter, the viscosity and surface tension of the bio-ink, the pneumatic pressure and the valve opening time [155]. It offers controlled deposition of materials via a layer-by-layer fabrication approach; the key advantages of microvalve-based bioprinting are the synchronized ejection of biomaterials and cells from different print-heads, deposition of thin material layer (1-2 μm thickness) and precise cellular positioning with high viability greater than 86% [182] and high throughput printing ($\sim 1,000$ printed droplets per second) [155]. However, it is only possible to print hydrogels within a limited range of viscosities (~ 1 to $200 \text{ mPa}\cdot\text{s}$) and cell concentration of up to 10^6 cells/ml due to the clogging issues in the small nozzle orifice ($100 - 250 \mu\text{m}$) [154, 155]. The cells tend to sediment over time, affecting the overall cell homogeneity within the bio-inks.

The valve opening time (VOT), printing pressure and the nozzle size are critical system parameters that determine droplet formation in a microvalve-based bioprinting system. As the viscosity increases, a longer VOT is necessary to generate the bio-ink droplet. However, a VOT that is higher than VOT_{max} will

induce the formation of satellite droplets [183]. Hence, there is an optimal range of VOT values [VOT_{min} , VOT_{max}] to achieve single droplet dispensing for each specific bio-ink [183]. A minimum printing pressure is required to provide an adequate force for droplet generation and this p_{min} increases with increasing fluid viscosity. When the pressure is below the minimum printing pressure, a huge droplet will start to accumulate on the nozzle orifice due to insufficient force to overcome the surface tension of fluid. In contrast, excessive printing pressure would result in formation of satellite droplets. The printing pressure has a huge influence on the cellular behavior; it was reported that cells that were exposed to an optimal printing pressure of less than 0.5 bars will not exhibit any detrimental short-term or long-term impairments [184]. Furthermore, it was reported that a variation in the nozzle size is a more effective approach to tune the droplet diameter, whereas a variation in printing pressure (0.15 – 0.4 bars) does not result in a significant change in droplet diameter [183]. Although the smallest nozzle diameter provides the highest printing resolution, it also has the narrowest range of optimal VOT values [183]. Hence, optimization of the printing process is necessary for high resolution microvalve-based printing. In the following sections, the modeling of the microvalve-based bioprinting process was performed. The printing process can be represented by 2 critical models, the shear stress model within the print-head and the droplet ejection and impact process respectively. The models indicated that the bio-ink properties play a critical role in affecting the printed cells. As such, further experiments are conducted to investigate the influence of bio-ink properties on the printed cells.

3.2. Modeling of microvalve-based bioprinting process

3.2.1. Shear stress model within the microvalve-based print-head

The geometry of the microvalve-based print-head in the proposed fluid dynamics model is simplified to a pipe-flow system with three sections: pipe (M1), annulus (M2) and nozzle (M3). The flow in each particular section can be represented by the *Bernoulli*-equation for unsteady flow, Equation 3.1.

$$P_x + \frac{1}{2}\rho v_x^2 + \rho gh_x = P_{x+1} + \frac{1}{2}\rho v_{x+1}^2 + \rho gh_{x+1} + \rho \int_0^{M_x} \frac{\partial v}{\partial t} dm + \Delta P(v, \eta)$$

(3.1)

$$M1: P_0 + \frac{1}{2}\rho v_0^2 + \rho gh_0 = P_1 + \frac{1}{2}\rho v_1^2 + \rho gh_1 + \rho \int_0^{L_1} \frac{\partial v_1}{\partial t} dL_1 + \Delta P_1(v_1, \eta) \quad (3.1.1)$$

$$M2: P_1 + \frac{1}{2}\rho v_1^2 + \rho gh_1 = P_2 + \frac{1}{2}\rho v_2^2 + \rho gh_2 + \rho \int_0^{L_2} \frac{\partial v_2}{\partial t} dL_2 + \Delta P_2(v_2, \eta) \quad (3.1.2)$$

$$M3: P_2 + \frac{1}{2}\rho v_2^2 + \rho gh_2 = P_3 + \frac{1}{2}\rho v_3^2 + \rho gh_3 + \rho \int_0^{L_3} \frac{\partial v_3}{\partial t} dL_3 + \Delta P_3(v_3, \eta) \quad (3.1.3)$$

The microvalve-based print-head is divided into three different sections. The liquid flow through each section can be represented by the *Bernoulli*-equation for unsteady flow (Equation 3.1.1 – 3.1.3). The equations can be summarized to Equation 3.2, assuming that the entrance speed (v_0) at the microvalve is insignificant and that the pressure (P_3) and the geodesic height (h_3) at the nozzle exit are zero. The pressure loss in the inlet area (ΔP_{inlet}) and the capillary pressure (ΔP_{cap}) were also accounted for in Equation 3.2.

$$P_0 + \rho gh_0 = \frac{1}{2}\rho v_3^2 + \rho \frac{\partial v_1}{\partial t} L_1 + \rho \frac{\partial v_2}{\partial t} L_2 + \rho \frac{\partial v_3}{\partial t} L_3 + \Delta P_1 + \Delta P_2 + \Delta P_3 + \Delta P_{inlet} + \Delta P_{cap} \quad (3.2)$$

Equation 3.2 can be converted into a non-linear, first order differential equation (Equation 3.7) that describes the flow of bio-ink through the different segments of the microvalve-based print-head considering inertia, wall friction and intrinsic shear stress by using the *Hagen-Poiseuille* Law (Equation 3.3), the *Power Law* (Equation 3.4) and the *Continuity Law* (Equation 3.5)

$$\Delta P(v, \eta) = 8 \frac{\dot{v} \eta L}{\pi r^4} \quad (3.3)$$

$$\eta = K \left(\frac{\delta v}{\delta r} \right)^{n-1} \quad (3.4)$$

$$v_a = \frac{A_b}{A_a} v_b \rightarrow v_1 = \frac{A_3}{A_1} v_3 \text{ and } v_2 = \frac{A_3}{A_2} v_3 \quad (3.5)$$

Rearranging Equation 3.2 and substituting Equation 3.5 to obtain Equation 3.6:

$$\rho \frac{\partial v_1}{\partial t} L_1 + \rho \frac{\partial v_2}{\partial t} L_2 + \rho \frac{\partial v_3}{\partial t} L_3 = P_0 + \rho g h_0 - \frac{1}{2} \rho v_3^2 - (\Delta P_1 + \Delta P_2 + \Delta P_3 + \Delta P_{inlet} + \Delta P_{cap})$$

$$\rho \frac{A_3}{A_1} \frac{\partial v_3}{\partial t} L_1 + \rho \frac{A_3}{A_2} \frac{\partial v_3}{\partial t} L_2 + \rho \frac{\partial v_3}{\partial t} L_3 = P_0 + \rho g h_0 - \frac{1}{2} \rho v_3^2 - (\Delta P_1 + \Delta P_2 + \Delta P_3 + \Delta P_{inlet} + \Delta P_{cap})$$

$$\frac{\partial v_3}{\partial t} = \frac{1}{\rho A_3 \left(\frac{L_1}{A_1} + \frac{L_2}{A_2} + \frac{L_3}{A_3} \right)} \left[P_0 + \rho g h_0 - \frac{1}{2} \rho v_3^2 - (\Delta P_1 + \Delta P_2 + \Delta P_3 + \Delta P_{inlet} + \Delta P_{cap}) \right] \quad (3.6)$$

$$\Delta P_1 = v_3^n \frac{A_3}{A_1} \frac{4}{d_1} L_1 K \left[\frac{2 \left(\frac{1}{n} + 3 \right)}{d_1} \right]^n \quad (3.6.1)$$

$$\Delta P_2 = v_3^n \frac{A_3}{A_2} \frac{4}{d_2} L_2 K \left[\frac{2 \left(\frac{1}{n} + 3 \right)}{d_2} \right]^n \quad (3.6.2)$$

$$\Delta P_3 = v_3^n \frac{4}{d_3} L_3 K \left[\frac{2 \left(\frac{1}{n} + 3 \right)}{d_3} \right]^n \quad (3.6.3)$$

$$\Delta P_4 = \Delta P_{inlet} = \frac{1}{2} \rho v_3^2 \frac{A_3}{A_1} \left(\frac{d_1}{d_3} \right)^4 \quad (3.6.4)$$

$$\Delta P_5 = \Delta P_{cap} = \frac{2 \sigma_{surface}}{0.5 d_3} \quad (3.6.5)$$

$$\frac{\partial \bar{v}}{\partial t} = \frac{1}{\rho A_3 (\frac{L_1}{A_1} + \frac{L_2}{A_2} + \frac{L_3}{A_3})} [P_0 + \rho g h_0 - \frac{1}{2} \rho \bar{v}^2 - \sum_{i=1}^5 \Delta P_i(\bar{v}, \eta)] \quad (3.7)$$

The power-law constants (K and n) for the shear-thinning fluid in the fluid dynamics model can be obtained experimentally from the rheological measurements (log-values of shear stress over the shear rate). The flow consistency index (K) can be determined from the y-axis intercept whereas the flow behavior index (n) is indicated by the slope of the graph. The average flow rate inside the nozzle over time for different bio-inks, nozzle diameters and printing pressure can be calculated numerically from Equation 3.7.

Using the numerically derived average flow rate, the velocity and shear stress profile in the microvalve-based print-head could be calculated analytically. The velocity profile in the nozzle can be determined according to Equation 3.8 by combining the *Stokes' Law* and *Power Law* (3.4).

$$v(r) = \bar{v} \left(\frac{3 + \frac{1}{n}}{1 + \frac{1}{n}} \right) \left[1 - \left(\frac{r}{r_o} \right)^{\frac{n+1}{n}} \right] \quad (3.8)$$

$$\tau = K D^n = K \left(-\frac{\partial v}{\partial r} \right)^n \quad (3.9)$$

$$\frac{\partial v}{\partial r} = -\bar{v} \left(3 + \frac{1}{n} \right) \frac{r^{\frac{1}{n}}}{r_o^{\frac{n+1}{n}}} \quad (3.10)$$

$$\tau = K \left[\frac{\bar{v}}{r_o} \left(3 + \frac{1}{n} \right) \right]^n \frac{r}{r_o} \quad (3.11)$$

The resulting shear stress profile (Equation 3.11) can be represented by the *Power Law* (Equation 3.9) and shear rate (Equation 3.10), which could be derived from the velocity (Equation 3.8). The derived equation can be used to determine the shear stress profile within the nozzle; with higher shear stress near the wall of the nozzle [184].

3.2.2. Droplet ejection and impact process

The four key parameters that influence the printability include viscosity, density and surface tension of the printable bio-inks and the radius of printing orifice [185]. The printability of the bio-inks can be denoted by the Reynolds number (N_{Re} : a ratio of inertial to viscous forces) and the Weber number (N_{We} : a balance between the inertial and capillary forces).

$$N_{Re} = \frac{vr\rho}{\eta} \quad (3.12)$$

$$N_{We} = \frac{v^2r\rho}{\gamma} \quad (3.13)$$

$$Z = \frac{N_{Re}}{(N_{We})^{1/2}} = \frac{(r\rho\gamma)^{1/2}}{\eta} \quad (3.14)$$

where v , ρ , η and γ are the average travel velocity, density, viscosity and surface tension of the bio-inks respectively, and r is a characteristic dimension (radius of the printing orifice). The Z value is the inverse of Ohnesorge number (Oh), which can be represented as a ratio between the Reynolds number and square root of the Weber number and it is independent of bio-ink velocity.

Besides being subjected to the shear stress within the printing nozzle, the droplet impact during the printing process also influences the viability of printed cells. After the cell-containing droplet is ejected from the microvalve-based print-head, the droplet impact process can be further divided in four distinct stages: 1.Before impact, 2.Maximum spread, 3.Maximum recoil/rebound and finally 4.Equilibrium. The total energy is conserved in the system defined by a control volume that comprises a liquid droplet, a substrate surface and the surrounding environment. The energy level of the substrate surface can be assumed to be zero as a reference for the system.

Right before impact, the total kinetic energy (KE_1) and total surface energy (SE_1) of a spherical droplet can be represented by:

$$KE_1 = (\frac{1}{2}\rho v_o^2)(\frac{\pi}{6}D_o^3) \quad (3.15)$$

$$SE_1 = \pi D_o^2 \gamma \quad (3.16)$$

After impact, the droplet reaches a maximum spreading diameter, D_{max} . At the maximum spread, the flattened droplet is assumed to be a thin circular disk [186]. Using the volume conservation law, the top surface area of the flattened droplet is $\frac{\pi}{4}D_{max}^2$ and the maximum height of the flattened droplet is $\frac{2}{3}\frac{D^3}{D_{max}^2}$. The surface energy is the sum of the liquid-vapour surface energy and the solid-liquid surface energy minus the solid-vapour surface energy which has been lost in the process:

$$SE_2 = SE_{LV} + SE_{SL} - SE_{SV} = \frac{\pi}{4}D_{max}^2(\gamma_{LV} + \gamma_{SL} - \gamma_{SV}) \quad (3.17)$$

The Young's equation can be used to relate the difference $\gamma_{SL} - \gamma_{SV}$ to γ_{LV} and static contact angle θ :

$$\gamma_{LV}\cos\theta = -(\gamma_{SL} - \gamma_{SV}) \quad (3.18)$$

$$\therefore SE_2 = \frac{\pi}{4}D_{max}^2(1 - \cos\theta)\gamma_{LV} \quad (3.19)$$

The work done in deforming the droplet against viscosity can be represented by [187]:

$$W = \int_0^{t_s} \int_V \phi \, dV dt \approx \phi V t_s \quad (3.20)$$

where V is the volume of the viscous fluid, t_s is the time taken for the droplet to spread and ϕ is the viscous dissipation function. The dissipation function ϕ can be expressed as

$$\phi = \eta \left(\frac{\partial v_i}{\partial x_j} + \frac{\partial v_j}{\partial x_i} \right) \frac{\partial v_i}{\partial x_j} \approx \eta \left(\frac{dv}{dy} \right)^2 \approx \eta \left(\frac{v_o}{L} \right)^2 \quad (3.21)$$

where $\frac{dv}{dy}$ is the normal velocity gradient in the boundary layer. The volume of the viscous layer is $V = \frac{\pi}{4} D_{max}^2 \delta$, where δ is the boundary thickness. An analytical solution for the boundary layer thickness can be obtained by assuming that the liquid motion in the droplet can be denoted by the axisymmetric stagnation point flow. The stream-function (Ψ) for potential flow outside the boundary layer in such a flow is given as [188]

$$\Psi = -Br^2 y \quad (3.22)$$

where B is a constant. The liquid velocity component normal to the wall is given as $v_y = -2By$; assuming $v_y = -v_o$ at $y = D_o/2$ gives $B = v_o/D_o$. With the free-stream velocity distribution described by the stream-function of Equation 3.21, a similarity solution for the boundary layer thickness can be represented by:

$$\delta = 2 \frac{D_o}{\sqrt{NRe}} \quad (3.23)$$

The energy lost to viscous dissipation can be estimated by substituting Equation 3.21 and 3.23 into Equation 3.20, assuming that $L = \delta$, $t_s = 8D_o/3v_o$, $V = \frac{\pi}{4} D_{max}^2 \delta$, giving

$$W = \int_0^{t_s} \int_V \phi dV dt \approx \phi V t_s$$

$$W = \frac{\pi}{3} \rho v_o^2 D_o D_{max}^2 \frac{1}{\sqrt{N_{Re}}} \quad (3.24)$$

Using the energy conservation law, $KE_1 + SE_1 = KE_2 + SE_2 + W$ and combining Equation 3.15, 3.16, 3.19 and 3.20, a simple expression for the maximum droplet spreading factor, ξ could be obtained:

$$KE_1 + SE_1 = KE_2 + SE_2 + W$$

$$\left(\frac{1}{2} \rho v_o^2\right) \left(\frac{\pi}{6} D_o^3\right) + \pi D_o^2 \gamma = 0 + \frac{\pi}{4} D_{max}^2 \gamma (1 - \cos\theta) + \frac{\pi}{3} \rho v_o^2 D_o D_{max}^2 \frac{1}{\sqrt{N_{Re}}}$$

$$\pi D_o^2 \left(\frac{1}{12} \rho v_o^2 D_o + \gamma\right) = \pi D_{max}^2 \left[\frac{1}{4} (1 - \cos\theta) \gamma + \frac{1}{3} \rho v_o^2 D_o \frac{1}{\sqrt{N_{Re}}}\right]$$

$$\frac{D_{max}^2}{D_o^2} = \frac{\left(\frac{1}{12} \rho v_o^2 D_o + \gamma\right)}{\frac{1}{4} (1 - \cos\theta) \gamma + \frac{1}{3} \rho v_o^2 D_o \frac{1}{\sqrt{N_{Re}}}}$$

$$\frac{D_{max}}{D_o} = \sqrt{\frac{(\rho v_o^2 D_o + 12\gamma)}{3 (1 - \cos\theta) \gamma + 4 \rho v_o^2 D_o \frac{1}{\sqrt{N_{Re}}}}}$$

$$\frac{D_{max}}{D_o} = \sqrt{\frac{\left(\frac{\rho v_o^2 D_o}{\gamma} + 12\right)}{3 (1 - \cos\theta) + 4 \frac{\rho v_o^2 D_o}{\gamma} \frac{1}{\sqrt{N_{Re}}}}}$$

$$\therefore \xi = \frac{D_{max}}{D_o} = \sqrt{\frac{(N_{We} + 12)}{3 (1 - \cos\theta) + 4 \frac{N_{We}}{\sqrt{N_{Re}}}}} \quad (3.25)$$

Apart from the printing-induced damage to the cells at the printing nozzle, significant damage is also expected to occur during the impact of the cell droplets on substrate surface. Particularly, droplet impact generally induces cell damage as a result of cell membrane elongation and deformation [189].

The Gaussian distribution of cell diameter, D_c , can be represented as:

$$f(D_c) = \frac{1}{\sigma \sqrt{2\pi}} \exp \left[\frac{-(D_c - D_{c,avg})^2}{2\sigma^2} \right] \quad (3.26)$$

The cell shape is defined by assuming that cell deformation into an oblate spheroid using volume conservation law. At the instant of maximum extension, the height of the impacting cell can be expressed as $h_c = D_c^3 / D_{c,max}^2$. The deformation Ω of an impacting cell can be expressed as:

$$\Omega = \frac{D_{c,max} - h_c}{D_{c,max} + h_c} = \frac{D_{c,max}^3 - D_c^3}{D_{c,max}^3 + D_c^3} \quad (3.27)$$

Assuming that the cells have different probability of cell death for a given expansion and uniform expansion of cell membrane, a critical expansion Ω_{cr} for the tolerable limit of expansion was defined.

The viability of individual cell Z can be expressed as

$$Z(\Omega) = 1 \quad \text{for } \Omega < \Omega_{cr} - \Delta\Omega \quad (3.28)$$

$$Z(\Omega) = \frac{1}{2} - \frac{\Omega - \Omega_{cr}}{2\Delta\Omega} \quad \text{for } \Omega_{cr} - \Delta\Omega \leq \Omega \leq \Omega_{cr} + \Delta\Omega \quad (3.29)$$

$$Z(\Omega) = 0 \quad \text{for } \Omega > \Omega_{cr} + \Delta\Omega \quad (3.30)$$

3.3. Influence of bio-ink properties on viability of printed cells

A suitable bio-ink is developed using a neutral macromolecule, polyvinylpyrrolidone (PVP) [190], as it is one of the most prevalently-used excipients in pharmaceuticals. A microvalve-based bioprinter with multiple print-heads (50 μm nozzle radius) is utilized for this study; a constant printing pressure of 0.25 bars is used for all the PVP-based bio-inks as a prior study elucidated the harmful effect of high printing pressure (> 0.25 bars) on printed cells [184]. As a proof-of-concept, neonatal human foreskin fibroblast cell line (HFF-1 from ATCC® SCRC-1041TM) is used. In this work, PVP is employed to tune the physical properties (eg. viscosity, surface tension and density) of the bio-inks. Firstly, PVP-based bio-inks of different polymer concentration are formulated and validation tests are conducted to assess the performance of PVP-based bio-inks for cell bioprinting. The measurement results are consolidated in Table 3-1.

Table 3-1. Effect of varying polymer concentration on bio-ink properties and cell viability

PVP concentration	Cell concentration	Density (kg/m^3)	Viscosity (mPa.s)	Surface Tension (mN/m)	Nozzle radius (μm)	Z	Short-term viability (%)
0% w/v	1.0 mil cells/ml	1001.3 ± 3.9	0.85 ± 0.05	59.8 ± 0.2	50	64.36	80.1 ± 0.83
1% w/v	1.0 mil cells/ml	1009.3 ± 2.8	2.94 ± 0.03	51.5 ± 0.2	50	17.33	88.6 ± 0.83
2% w/v	1.0 mil cells/ml	1020.3 ± 2.7	5.29 ± 0.05	47.5 ± 0.2	50	9.30	92.4 ± 1.30
2.5% w/v	1.0 mil cells/ml	1025.3 ± 3.1	8.19 ± 0.14	43.2 ± 0.2	50	5.75	95.4 ± 1.04
3% w/v	1.0 mil cells/ml	1029.8 ± 3.2	12.43 ± 0.20	41.7 ± 0.2	50	3.73	-

The physical properties of the PVP-based bio-inks are measured; a higher PVP concentration has resulted in an increase in both viscosity and density, but a decrease in surface tension. Generally, a lower Z value is obtained with higher PVP concentration. The generic PVP-based bio-inks have a printable range of Z values within $5.75 \leq Z \leq 64.36$ (0 - 2.5% w/v) ; the 3% w/v PVP-based bio-ink with a Z value of 3.73 has demonstrated inconsistent printing as the lower limit of Z values is governed by the maximum printable viscosity of the bio-ink [185]. Based on results from the printability study, the viability of printed cells is evaluated using the generic PVP-based bio-inks (0 – 2.5% w/v). The data has indicated that immediate viability of printed cells increases with lower Z values (Fig 3-1) because of slower droplet velocity [185] and the additional cushioning effect (higher energy dissipation) from the more viscous bio-inks protect the printed cells [191, 192].

Fig 3-1. Effect of polymer concentration on Z values and respective cell viability directly after printing; scale bar: 200 μm .

Further study is performed to understand the effect of cell concentration on the physical properties of the PVP-based bio-inks and their respective cell viability. The 2.5% w/v PVP-based bio-inks are formulated using different cell concentration (0.5 - 2.5 million cells/ml) and an increase in cell concentration has resulted in lower Z values (Table 3-2). This is probably due to the turbulent fluid flow and frictional forces at the fluid-cell interface. The presence of more cells also decreases the surface tension and results in a drop in total free energy of the bio-ink. The results are corroborated by another study [193]; the change in cell concentration only resulted in slight variation in the Z values and there is no significant effect on the immediate cell viability (Fig 3-2 and Fig 3-3). An increase in the cell concentration beyond 2.0 mil cells/ml results in inconsistent printing.

Table 3-2. Effect of varying cell concentration on bio-ink properties and cell viability

PVP concentration	Cell concentration	Density (kg/m ³)	Viscosity (mPa.s)	Surface Tension (mN/m)	Nozzle radius (μm)	Z	Short-term viability (%)
2.5% w/v	0.5 mil cells/ml	1024.3 ± 2.3	8.08 ± 0.13	43.7 ± 0.2	50	5.85	95.4 ± 0.71
2.5% w/v	1.0 mil cells/ml	1025.3 ± 3.1	8.19 ± 0.14	43.2 ± 0.2	50	5.75	95.4 ± 1.04
2.5% w/v	1.5 mil cells/ml	1026.3 ± 2.4	8.26 ± 0.13	42.7 ± 0.2	50	5.67	95.9 ± 0.78
2.5% w/v	2.0 mil cells/ml	1026.2 ± 2.8	8.41 ± 0.14	42.3 ± 0.2	50	5.54	96.1 ± 0.82
2.5% w/v	2.5 mil cells/ml	1027.2 ± 2.7	8.65 ± 0.15	42.1 ± 0.2	50	5.38	-

Fig 3-2. Effect of cell concentration on Z values and the respective cell viability directly after printing;
scale bar: 200 μ m

Fig 3-3. Short-term cell viability directly after printing (mean $\pm$ SD). Significance levels are as follows: $p < 0.005$ (***) , $p < 0.05$ (*)

Table 3-3. Effect of varying polymer and cell concentration on bio-ink properties and the respective immediate cell viability

PVP concentration	Cell concentration	Density (kg/m ³)	Viscosity (mPa.s)	Surface Tension (mN/m)	Nozzle radius (μm)	Z	Short-term viability (%)
0% w/v	1.0 mil cells/ml	1001.3 ± 3.9	0.85 ± 0.05	59.8 ± 0.2	50	64.36	80.1 ± 0.83
1% w/v	1.0 mil cells/ml	1009.3 ± 2.8	2.94 ± 0.03	51.5 ± 0.2	50	17.33	88.6 ± 0.83
2% w/v	1.0 mil cells/ml	1020.3 ± 2.7	5.29 ± 0.05	47.5 ± 0.2	50	9.30	92.4 ± 1.30
2.5% w/v	0.5 mil cells/ml	1024.3 ± 2.3	8.08 ± 0.13	43.7 ± 0.2	50	5.85	95.4 ± 0.71
2.5% w/v	1.0 mil cells/ml	1025.3 ± 3.1	8.19 ± 0.14	43.2 ± 0.2	50	5.75	95.4 ± 1.04
2.5% w/v	1.5 mil cells/ml	1026.3 ± 2.4	8.26 ± 0.13	42.7 ± 0.2	50	5.67	95.9 ± 0.78
2.5% w/v	2.0 mil cells/ml	1026.2 ± 2.8	8.41 ± 0.14	42.3 ± 0.2	50	5.54	96.1 ± 0.82
2.5% w/v	2.5 mil cells/ml	1027.2 ± 2.7	8.65 ± 0.15	42.1 ± 0.2	50	5.38	-
3% w/v	1.0 mil cells/ml	1029.8 ± 3.2	12.43 ± 0.20	41.7 ± 0.2	50	3.73	-

The experimental data (Fig 3-3) indicate that the immediate cell viability is inversely proportional to the log Z values within the range of printable Z values ($5.54 \leq Z \leq 64.36$). The immediate cell viability increases significantly with bio-inks of lower Z values and this study highlights the significant effect of bio-ink properties on the immediate cell viability. Hence, a suitable PVP-based bio-ink with a low Z value (< 9.3) can be used to achieve enhanced cell viability ($> 90\%$) (Table 3-3).

The study is extended to evaluate the long-term cell viability (up to 96 hours) using AlamarBlue® assay. The number of viable cells at any particular time point can be represented by the normalized relative fluorescence units (RFUs) with respect to the 0-hour RFU values for each individual bio-ink to ensure a fair comparison across the different groups. The AlamarBlue® assay results indicate significant higher number of cells in both 2% and 2.5% w/v PVP-based bio-inks than 0% and 1% w/v PVP-based bio-inks at the 24- and 96-hour interval. A significant increase in the cell number between the 0% and 1% w/v PVP-based bio-inks is only observed at 96-hour interval. The results have also indicated that an increase in PVP concentration from 2% w/v to 2.5% w/v has no significant effect on the cell viability at both 24- and 96- hour interval. The use of a bio-ink with lower Z values generally results in enhanced cell viability; an optimal PVP-based bio-ink with a Z value threshold of ≤ 9.30 ($\geq 2\%$ w/v PVP) helps to protect the cells from significant printing-induced cell damage (Fig 3-4). The experimental results are corroborated by the cell viability model, an increase in the PVP concentration results in more viscous bio-ink which results in a lower maximum droplet spreading factor, ξ . Hence, an increase in PVP concentration results in less cell deformation and ultimately improves the overall viability of printed cells.

Fig 3-4. (Top) Fluorescence images of printed cells across varying time points; scale bar: 200 μ m, (Bottom) Analysis of the viability of printed cell across varying time points over a 96-hours period (mean $\pm$ SD). Significance levels are as follows: $p < 0.005$ (***), $p < 0.05$ (*).

3.4. Influence of bio-ink properties on homogeneity of printed cells

Different PVP-based bio-inks (0 – 2.5% w/v) are formulated using a constant cell concentration of 1mil cells/ml to determine and analyze the cell homogeneity during the drop-on-demand printing process over a 30-minute printing duration [194, 195] (Fig 3-5).

Fig 3-5. (Top) Fluorescence images of printed cells (at 1.0 mil cells/ml) across varying time points; scale bar: 200 μ m, (Bottom) Analysis of cell homogeneity over time (mean $\pm$ SD). Significance levels are as follows: $p < 0.005$ (***), $p < 0.05$ (*).

A relatively constant number of printed cells is observed in all the groups, followed by a gradual increase in the number of printed cells from 0- to 15-minute interval and lastly a decrease in the number of printed cells from 20- to 30-minute. This phenomenon can be explained by the sedimentation effect that occurs as a result of gravitational forces acting on the floating cells. An increase in the PVP concentration alleviates the sedimentation effect due to the increase of bio-ink density. The most significant cell sedimentation effect could be observed in the 0% w/v PVP bio-ink (Fig 3-5), whereby a huge increase in the number of printed cells is observed over time. Little information on the cell homogeneity during the printing process is known, it is a complex phenomenon that has yet to be resolved. A previous study utilized *in silico* testing to analyze the cell output during the printing process [165]; the model predicted a linear increase in cell output prior to a constant steady-state cell output. The stark difference between the experimental and simulation data suggests the complexity of this issue. As such, further study is performed to investigate the inconsistent cell output throughout the printing process.

Prior studies have highlighted the issue of cell adhesion on the surface of printing cartridges due to the van der Waals interactions [196, 197]. The adhered cells on the cartridge surface lead to the formation of agglomeration of cell aggregates at the constriction of printing cartridge. The incorporation of higher PVP concentration alleviates the cell sedimentation effect and hinders cell adhesion on the cartridge surface during the printing process. The pre-coating of the interior surface within printing cartridges is performed using 2.5% w/v PVP solution overnight at 4⁰ C and the cell output for 0% w/v PVP-based bio-ink in both the coated and non-coated printing cartridges are evaluated over a period of 30 minutes (Fig 3-6). The experimental data reveal that a more consistent cell output over time in the coated printing cartridge as compared to the non-coated printing cartridge. The PVP coating hinders cell adhesion [198] and enhances the cell output consistency over time. As shown in the non-coated cartridge, the issue of

cell adhesion is evident over time and leads to a drastic decrease in the cell output when the printing duration is longer than 15 minutes.

Fig 3-6. (Top) Fluorescence images of printed cell droplets over time in both non-coated and coated printing cartridges at varying time points; scale bar: 200 μ m. (Bottom) Analysis of cell output over time (mean $\pm$ SD). Significance levels are as follows: $p < 0.005$ (***), $p < 0.05$ (*).

3.5. Discussion

The printed cells in the PVP-based droplets experience shear stress at the nozzle orifice and droplet impact upon hitting the substrate surface. The modeling of microvalve-based bioprinting process highlights the significant influence of bio-ink properties on the printed cells. As such, more experiments are conducted to understand the influence of bio-ink properties on printed cells. The two major challenges in bioprinting process include the poor cell viability and homogeneity. In this study, the PVP molecules are used as a polymer model to investigate the influence of bio-ink properties on the printed cells. The incorporation of PVP molecules within the bio-inks induces a change in the Z value (viscosity, surface and density) and affected the bio-ink printability. In this work, the printability of the bio-inks is determined to be within a range of Z values ($5.54 \leq Z \leq 64.36$). A bio-ink with higher PVP concentration (lower Z value) results in slower droplet velocity and offers extra cushioning effect (higher energy dissipation) for the printed cells to increase the cell viability during the printing process. The results also indicate that a change in cell concentration (from 0.5 mil cells/ml to 2.0 mil cells/ml) has no significant effect on the Z values and its corresponding cell viability. The printed cells that are cultured over a period of 96-hours, do not exhibit significant printing-induced damage when the Z values of the PVP-based bio-inks are below 9.30. Furthermore, a higher PVP concentration also alleviates the issue of cell sedimentation and adhesion inside the printing cartridge during the printing process. This study elucidates the significant effect of bio-ink properties on viability and homogeneity of printed cells during the DOD bioprinting process and offered critical information for the development of new printable bio-inks for enhanced cell viability and homogeneity. An analytical model has been developed to correlate the relationship between the influence of bio-ink properties and viability of printed cells using PVP-based bio-ink.

4. Engineering the complex 3D microenvironment in collagen-fibroblast matrices

4.1. Macromolecular crowding (MMC): a tool to modulate complex 3D microenvironment

Most native tissues comprise hierarchical porous structures [199] that play important roles in regulating cellular behavior. Collagen type I, which makes up the bulk of native tissue, provides structural stability and strength to various tissues such as skin, bone and even cartilage. Due to its well-characterized structure as an extracellular matrix protein, collagen type I has been prevalently used for emulating tissue properties and regulating cellular functions [200]. Over the years, conventional approaches for fabrication of 3D porous scaffolds such as freeze drying, phase separation and electrospinning have limited control and consistency over the scaffold architecture [201]. Macromolecular crowding (MMC) [172, 176, 178, 179] has emerged as an attractive strategy to manipulate the architecture of 3D matrices; the macromolecules exert excluded volume effect (EVE) on the surrounding biopolymer (e.g. proteins and nucleic acids) to emulate the physiological crowdedness found in the native tissues. The macromolecules in MMC can be represented by a parameter known as fractional volume occupancy (FVO) to evaluate the effectiveness of the macromolecular crowders in a given volume (which is dependent on the hydrodynamic radii of the crowders used). The physiological range of FVO within the human body is calculated to be within the range of 0 – 54% v/v. A recent study demonstrated that the addition of macromolecules into the culture media at optimal conditions amplified the ECM deposition within a 2D cell culture system [176]. Another study showed that polyvinylpyrrolidone (PVP) could be used to achieve a significantly higher FVO (up to 54% FVO, which is not achievable with previously studied macromolecules) and significant ECM deposition in a dose-dependent manner within a 2D cell culture system [178]. Besides applying MMC to enhance the ECM deposition within a 2D cell culture

system, MMC can also be applied to tune the architecture of 3D collagen hydrogels using Ficoll 400 macromolecules [179] but only up to 8% FVO. The presence of biological macromolecules in intracellular and extracellular environments is clearly obvious but more in-depth investigations are required to fully understand the phenomenon [172].

The 3D microenvironment of the dermal skin region has a highly-complex 3D architecture; different strategies have been applied to tune the 3D microenvironment of the collagen-based matrix to emulate the native skin. The skin dermal region serves as a critical foundation that provides critical signaling cues that regulate the skin homeostasis, understanding the influence of complex 3D microenvironment on cellular behavior would be critical for the development of 3D pigmented human skin constructs. Prior works have demonstrated that the macromolecular crowding (MMC) is an attractive strategy to alter the collagen fibrillogenesis and cellular behavior. In this work, the pre-fibrillated collagen solutions are supplemented with PVP macromolecules to fabricate 3D collagen-fibroblast matrices of different fractional volume occupancy (FVO) within the physiological range of 0 – 54 % FVO using conventional casting approach and in-depth characterization is performed to understand the synergistic effect of MMC on the 3D collagen-fibroblast matrices in term of cell-matrix remodeling and cellular behavior over a period of 7 days.

4.2. Influence of MMC on collagen fibrillogenesis process

To fully understand the influence of MMC on collagen fibrillogenesis, a research-grade pH/conductivity meter (Fisher Scientific, Accumet Research AR20) is used to determine the pH and ionic strength of the collagen solution at varying FVO (0 – 54 %) as these two key parameters were known to alter collagen fibrillogenesis. Fig 4-1A and Fig 4-1B present the change in pH and ionic strength as a function of varying FVO. There is no significant change in the pH from 7.25 ± 0.02 in 0 % FVO to 7.23 ± 0.02 in 54 % FVO. In addition, there is only slight increase in ionic strength from 150.1 ± 2.6 in 0 % FVO to 158.7 ± 1.6 in 54 % FVO. Therefore, increasing FVO has no significant effect on both the pH and ionic strength. As reported in a previous study, this negligible change in both the pH and ionic strength does not contribute significantly to any alteration in the collagen fibrillogenesis process [202].

To understand the influence of MMC on collagen formation kinetics, the collagen-fibroblast solutions are prepared at varying FVOs (0 – 54 %) and are transferred to a 96-well plate (100 μ l per well). The collagen assembly is monitored at 37 °C by a time-lapse measurement of the light absorbance at 313 nm using a Tecan Infinite M200 spectrophotometer and analysis of the collagen formation kinetics is performed by measuring the lag phase (t_{lag}) and the half-time ($t_{1/2}$) prior to complete collagen fiber assembly (Fig 4-1C). Collagen-fibroblast matrix scaffolds were successfully prepared at all tested crowding conditions, with varying fractional volume occupancy (FVO) ranging from 0 – 54 %. A PVP concentration of up to 54% FVO is selected to match the physiological range of fractional volume occupancy (FVO) found in the native tissues [177]. The rate of collagen I fibril formation is then quantified using a turbidity test at an absorbance of 313 nm over a period of 30 minutes (at every 1 minute interval) at a constant temperature of 37 °C (crosslinking temperature in the incubator). The

collagen assembly can be determined via turbidity measurement as the assembled collagen fibers absorb light at 313 nm.

Fig 4-1. Influence of MMC on A) pH and B) ionic strength at varying FVO (0 – 54%). C) Turbidimetric fibrillogenesis kinetics of collagen-based matrices under influence of varying FVO (0 – 54 %). D) A summary table on effect of MMC on pH, ionic strength and collagen formation kinetics.

The collagen nucleation (lag phase) is indicated by the 313 nm absorbance at a value close to zero, following which the absorbance increased significantly during the fibrillogenesis (growth phase) until it has reached a final plateau which represents the complete fiber assembly. Under all crowding conditions, a typical sigmoidal curve is observed with a lag phase (nucleation) having absorbance values close to zero, a growth phase (fibrillogenesis) and a final plateau corresponding to the complete fiber assembly. A significant decrease in the lag phase (t_{lag}) is measured at 54 % FVO as compared to the control 0 % FVO group (a significant decrease from ~ 9.2 min to ~ 0 min). Generally, the t_{lag} decreases with increasing PVP concentration. Macromolecular crowding also influences the $t_{1/2}$ with a significant decreased time for all the tested PVP concentration as compared to the control group (from 14.4 ± 0.3 min in 0 % FVO to 1.5 ± 0.4 min in 54 % FVO). As shown in the experimental results, macromolecular crowding has a significant effect on the fiber formation and fibrillogenesis kinetics in a dose-dependent manner.

An in-depth evaluation of the collagen-fibroblast matrices is performed using field-emission scanning electron microscopy (FE-SEM) to analyze the microstructure within the 3D matrices (Fig 4-2). The samples are first dehydrated using graded ethanol solutions (25%, 50%, 75%, 95% and lastly 100% ethanol for 20 minutes each). Next, the samples are placed in a critical point dryer from Leica EM CPD030, Germany and flushed with cold liquid CO_2 at $4^\circ C$ over a period of 2 hours to preserve the nanostructure within the 3D collagen matrices. The dried samples are then carefully sectioned using sterile surgical blade to expose the cross-sectional area of the collagen-based constructs. The cross-sectional area of the specimens are mounted onto aluminium stubs and coated with platinum (Pt) using Polarin SC7640 Sputter Coater from Quorum Technologies, United Kingdom. The cross-sections are examined with Ultra-Plus Field Emission Scanning Electron Microscope (FE-SEM) from Carl Zeiss, Germany. The electron images are taken at 2 keV accelerating voltage. Lastly, ImageJ analysis are performed on the FE-SEM images to evaluate the fiber diameter and pore size within the 3D collagen-

fibroblast matrices at varying FVOs (0 – 54 %). FE-SEM imaging (Fig 4-2A) is conducted ($n = 4$ for each FVO at different time points) and the analysis results are presented as mean $\pm$ standard deviation.

Fig 4-2. (A) SEM images of collagen-fibroblast matrices at different FVO (0 – 54 %); scale bar 1 μm . Influence of MMC on B) collagen fiber diameter and C) pore size (mean $\pm$ SD). D) Distribution of pore size in collagen-fibroblast matrices at different FVO (0 – 54 %). Significance levels are as follows: $p < 0.001$ (***), $p < 0.01$ (*).

The microstructure of the matrix scaffold is vastly altered by supplementing the pre-fibrillated ECM solution with increasing FVO (0 – 54 %) as shown in Fig 4-2A. The collagen matrices that are assembled in the standard condition (0 mg/ml PVP) have ECM networks characterized by thin and highly interwoven fibers with an overall homogeneous fiber matrix density, whereas the matrices that are prepared under macromolecular crowding show a looser fiber meshwork (indicated by highly-porous structure with thicker fibers) with increasing PVP concentration. A significant increase in collagen fiber diameter is only observed when a high 54 % FVO is used as compared to other groups. In contrast, a huge significant change in pore size is observed across all the groups ($0.45 \pm 0.09 \mu\text{m}$ in 0 % FVO, $0.67 \pm 0.10 \mu\text{m}$ in 18 % FVO, $0.98 \pm 0.12 \mu\text{m}$ in 36 % FVO and $1.67 \pm 0.26 \mu\text{m}$ in 54 % FVO). Hence, the results indicate that MMC plays a critical role in modulating the pore size of the 3D collagen-fibroblast matrices in a dose-dependent manner.

4.3. Influence of MMC on cell-matrix remodeling over time

In this work, 12-well insert plates are used to prepare the 3D collagen-based constructs. The resultant matrix consists of rat tail collagen type I (3.34 mg/ml) from Corning®, USA (70% of total volume), fibroblast growth medium from PromoCell, Germany (10% of total volume), polyvinylpyrrolidone (MW: 360 kDa) from Sigma-Aldrich, USA which is dissolved in 1x PBS solution (10% of total volume), normal human dermal fibroblasts (1.5 mil cells/ml) in fibroblast growth medium from Promocell, Germany (10% of total volume). The resultant pre-fibrillated collagen-fibroblast solution is then neutralized with 1M NaOH to pH 7; 350 µl of final collagen-fibroblast solution is added to each of the 12-well inserts (final collagen concentration of 2.4 mg/ml and cell concentration of 0.15 mil cells/ml) and incubated at 37 °C for an hour inside an incubator prior to addition of 1 ml fibroblast growth medium (Promocell, Germany) to the bottom of the culture insert (n = 36 for each FVO). The fibroblast growth medium is supplemented with 100 µM ascorbic acid (Sigma-Aldrich, USA) from Day 2 onwards and a constant medium change is performed once every 2 days.

To understand the influence of MMC on cell-matrix remodeling process, characterization is performed to evaluate the matrix contraction and change in microstructure of the 3D collagen-fibroblast matrices. The fabricated 3D collagen-fibroblast matrices are measured across varying time intervals (Day 1, Day 4 and Day 7) to evaluate the matrix contraction (in term of % remaining surface area) (Fig 4-3). To further understand this phenomenon at the nano-scale level, FE-SEM imaging (Fig 4-4) are performed across varying time intervals (Day 1, 4 and 7) to determine the change in the architecture of the 3D collagen-

fibroblast matrices. The sample preparation is discussed in the earlier section and all the images are analyzed using ImageJ software and presented as mean $\pm$ standard deviation.

Fig 4-3. Influence of MMC on matrix contraction. (A) Representative images of collagen-fibroblast matrices with different FVO (0 – 54 %); scale bar: 1 cm and (B) analysis of matrix contraction at different time points over a period of 7 days. Significance levels are as follows: $p < 0.001$ (***), $p < 0.01$ (*).

Fig 4-4. (A) Influence of MMC on microstructure of 3D collagen-fibroblast matrices and (B) analysis of change in pore size over a period of 7 days. Significance levels are as follows: $p < 0.001$ (***), $p < 0.01$ (*).

The collagen-fibroblast matrices are known to undergo a matrix contraction over time due to the cell-matrix remodeling process; however the mechanism behind this contraction phenomenon is still poorly understood. In this work, investigation is conducted to elucidate the effect of MMC on the cell-matrix remodeling process by evaluating the matrix contraction at different FVO (0 – 54 %) and analyzing the change in the microstructure of collagen-fibroblast matrices over a period of 7 days. No significant matrix contraction across all sample groups (0 – 54 % FVO) is observed on Day 1 (Fig 4-3B). There is only minimal contraction for both 18 % and 36% FVO samples ($96.3 \pm 1.4\%$ and $98.2 \pm 0.7\%$ respectively), whereas significant matrix contraction is observed in the control samples (0 % FVO, 90.7 ± 1.0 % of total surface area) and 54 % FVO samples (82.4 ± 1.2 % of total surface area) at Day 4. There is significant matrix contraction across all sample groups (0 – 54 % FVO) at Day 7. The samples in 18% and 36% FVO groups are reduced to 91.2 ± 1.9 % (*) and 93.1 ± 2.0 % (*) of the total surface area respectively, whereas the samples in 0% and 54% FVO groups are further reduced to 57.6 ± 4.2 % (***) and 51.5 ± 3.6 % (***) of the total surface area respectively. Furthermore, FE-SEM images are captured at different time points over a period of 7 days to investigate the influence of MMC on the cell-matrix remodeling (within collagen-fibroblast matrices) at the nano-level scale (10,000x magnification). The experimental data indicate that the average pore size in 0 – 36 % FVO groups increases over time (from Day 1 to Day 7), whereas the average pore size in 54 % FVO group generally decreases over time (from 1.67 ± 0.26 μm in Day 1 to 1.13 ± 0.17 μm in Day 7).

4.4. Influence of MMC on cellular behavior

In this section, work is conducted to investigate the influence of MMC on cell proliferation, cell morphology and ECM secretion within the 3D collagen-fibroblast matrices. Prior work on MMC mainly focused on 2D cell culture systems and this study investigates the influence of MMC on cell proliferation, cell morphology and ECM secretion within the 3D collagen-fibroblast matrices.

AlamarBlue® cell viability reagent is used to semi-quantify the cell proliferation rate by measuring the relative fluorescence units (RFUs) using a Tecan Infinite M200 spectrophotometer (n = 4 for each FVO at different time points). The cell proliferation rate can be quantified based upon the RFUs, whereby an increase in cell number is represented by a higher RFU (Fig 4-5A).

FE-SEM imaging are also performed to characterize the change in cell morphology (Fig 4-5B) within the 3D matrices following similar sample preparation steps as mentioned in earlier sections. All the FE-SEM images are analyzed using ImageJ software and presented as mean $\pm$ standard deviation.

The 3D collagen-fibroblast matrices (0 – 54 % FVO, n = 4 for each FVO at different time points) are embedded in Tissue-Tek® optimum cutting temperature (OCT) and snap frozen in liquid nitrogen prior to cryostat sectioning using a research cryostat (Leica CM3050 S) at -22°C . The 10 μm cryosections are then fixed in a mixture of methanol and acetone (in a 1:1 ratio) at -20°C for 20 minutes, followed by air-drying in a fume hood. The dried samples are then washed with tris buffered saline (TBS) supplemented with 0.1 % triton-X (washed thrice, 5 minutes per wash at room temperature). Non-specific binding of antibodies is blocked using 10 % fetal bovine serum (heat inactivated) in tris buffered saline (TBS) supplemented with 0.1 % triton-X for an hour at room temperature and the samples are incubated with

anti-fibronectin antibody (Abcam; dilution 1:100), anti-laminin 5 antibody (Abcam; dilution 1:100), anti-collagen IV antibody (Abcam; dilution 1:100) and anti-collagen VII antibody (Abcam; dilution 1:100) at 4 °C overnight. The samples are then washed with tris buffered saline (TBS) supplemented with 0.1 % triton-X (washed thrice, 5 minutes per wash at room temperature). Secondary antibodies (Alexa Fluor 488 and 568 from Abcam; dilution 1:1000) are then added for an hour at room temperature. Cell nuclei are counterstained using VectaShield Hardset™ with DAPI. The samples are then left to dry for 24 hours prior to visualization using Olympus fluorescent microscope (Fig 4-6B).

To evaluate the cell proliferation assay within the 3D matrices, Alamarblue® cell viability reagent is used to analyze the cell growth based on the fluorescence readout in the microplate reader. The number of living cells can be represented by the relative fluorescence units (RFUs) normalized to the control group (0 % FVO at Day 1). It is observed that a higher FVO generally results in a significant decrease in normalized RFUs over a period of 7 days (with increasing FVO) as shown in Fig 4-5A. This suggests that a higher FVO generally results in a slower cell growth within the 3D collagen-fibroblast matrices. Fig 4-5B reveals the cell morphology of the fibroblasts within the 3D matrices of varying FVO (0 – 54 %); the fibroblast lineages can be determined by the cell morphology and fibroblasts from different lineages are shown to exhibit different cellular behaviors [203]. It is observed that the fibroblasts within the 3D collagen matrices of lower FVO (0 – 18 %) generally exhibit bipolar morphology whereas the fibroblasts within the 3D collagen matrices of higher FVO (36 – 54 %) generally exhibit multi-polar morphology.

Fig 4-5. Influence of MMC on (A) cell proliferation, (B) cell morphology within 3D collagen-fibroblast matrices at varying FVO (0 – 54 %); scale bar: 10 μ m.

Signs of extracellular (ECM) deposition in the form of ultra-fine fiber-like meshes around the collagen fibers are observed near the cell filopodia, whereas the presence of significantly larger pores around the cell lamellipodia (Fig 4-6A) clearly suggests that matrix metalloproteinase (MMP) are secreted from the lamellipodia to cleave the surrounding ECM. To identify the type of ECM deposition within the 3D collagen-fibroblast matrices, immunofluorescence staining is performed to test for fibronectin, collagen IV, collagen VII and laminin-5 deposition. There is only positive staining for fibronectin, while negative staining is observed for collagen IV, collagen VII and laminin-5 deposition. The fibronectin deposition is calculated based on the area for positive staining (red) normalized to the 0 % FVO sample at Day 1 (Fig 4-6B); the fibronectin deposition is significantly higher with increasing FVO over a period of 7 days in Fig 4-6B and 4-7B. Particularly, significant fibronectin deposition is observed from Day 1 – 4 as compared to Day 4 – 7. Interestingly, the average number of filopodia per cell increases with increasing FVO and it strongly suggests that the cell filopodia could be the main site for ECM secretion.

Fig 4-6. A) Representative image of cell spreading within 3D collagen-fibroblast showing signs of ECM deposition around the cell filopodia and MMP secretion around the cell lamellipodia. B) Representative fluorescence images of fibronectin deposition in red counterstained with DAPI; scale bar: 100 μ m.

Fig 4-7. Analysis of A) number of filopodia per cell and B) fold change in fibronectin deposition at varying FVO (0 – 54 %). Significance levels are as follows: $p < 0.001$ (***), $p < 0.01$ (*).

4.5. Optimal design for 3D collagen-fibroblast matrices: hierarchical porous structures

In this earlier sections, the influence of MMC on cell-matrix remodeling and cellular behavior in 3D collagen-fibroblast matrices with varying FVO (0 – 54 %) was evaluated. The experimental results demonstrate that MMC plays a significant role in altering the pore sizes within the collagen-fibroblast matrices under different physiological conditions from 0 – 54 % FVO. Increasing MMC not only accelerates the collagen fibrillogenesis process, it also increases the pore size within the 3D matrices. Hence, MMC is an attractive approach to tune the pore size within the 3D collagen constructs.

These structural differences due to MMC lead us to examine the influence of physical properties of fibrillary microenvironment on cell-matrix remodeling and ECM secretion. Normal human dermal fibroblasts, the commonly-used skin cell type in dermal constructs, are seeded at an optimal cell concentration [158] to fabricate 3D collagen-fibroblast matrices under different FVOs (0 – 54 %). Matrix contraction is a prevalent phenomenon during tissue maturation; the experimental results show that minimal contraction is observed for both 18 % and 36 % FVO groups (~ 10 % contraction) whereas significant contraction is observed for both 0 % and 54 % FVO groups (~ 50 % contraction) over a period of 7 days. Hence, an optimal pore size for minimal matrix contraction is within the range of 0.67 – 0.98 μm . Further in-depth FE-SEM analysis of the cell-matrix remodeling at the nano-scale level reveals a significant increase in pore size of the 3D matrices for 0% FVO and 18 % FVO groups, no significant change in pore size for 36 % FVO group and a significant decrease in pore size for 54 % FVO group. It is reasonable to expect that the significant matrix contraction (in 0 % and 54 % FVO) would led to a decrease in pore size, however an unexpected increase in pore size is observed in 0% FVO samples. A closer look at the cell proliferation profile elucidates that significant higher cell proliferation rate is observed in 3D matrices of lower FVO, which could be due to the presence of smaller pore sizes that enhances the cell proliferation rate [145]. The significant increase in fibroblasts within the 0 % FVO

samples over the period of 7 days would in turn result in a marked increase of MMP secretion [204] during the cell-matrix remodeling process. This collagen cleavage process due to MMP secretion (which diffuses across the 3D matrices) is more dominant than the matrix contraction by the fibroblasts and the resultant effect is depicted by the increase in pore size over time (despite significant matrix contraction).

Another interesting phenomenon is the distinct differences in cell morphology across the samples. Most of the cells in the 3D matrices of higher FVO (bigger pore sizes) exhibit multi-polar morphology, whereas most of the cells in the 3D matrices of lower FVO (smaller pore sizes) exhibit bipolar morphology. This stark difference in cell morphology at different FVOs leads to differentiation along different fibroblast lineages (expressing different cellular behaviors).[203] Enhanced fibronectin deposition (an important ECM protein for growth factor sequestering and signaling[205]) is observed in the 3D collagen-fibroblast matrices of higher FVO (with bigger pore size and multi-polar cell morphology) at all time points over a period of 7 days. An optimal duration for dermal construct maturation is 4 days, as further culture (from Day 4 to Day 7) does not result in very significant ECM deposition statistically. The experimental results in the earlier section suggest that the filopodia could be the main site for ECM deposition (as indicated by signs of ultra-fine fiber-like meshes around the collagen fibers are observed near the cell filopodia), whereas the lamellipodia could be the main site for MMP secretion (as indicated by presence of significantly larger pores around the cell lamellipodia clearly suggested that MMPs are secreted from the lamellipodia and diffused outward to cleave the surrounding collagen fibers). The experimental results also highlight the need for co-culture (with epithelial cells such as keratinocytes) for the secretion of important ECM proteins such as collagen IV, collagen VII and laminin-5 (negative stain in the fabricated dermal constructs over a period of 7 days) that are found between the epithelial-dermal junctions within native skin tissue.

Most importantly, the experimental results highlight that significance of hierarchical pore sizes within the 3D collagen-fibroblast matrices. The 3D matrices of 0 % FVO (densely-packed matrix with small pore sizes) enhance cell growth but hindered ECM deposition, the 3D matrices of 18 – 36 % FVO (matrix with moderate pore sizes) experience minimal matrix contraction and exhibit moderate cell growth and ECM deposition, lastly the 3D matrices of 54 % FVO (loosely-packed matrix with large pore sizes) demonstrate slow cell growth but significantly higher ECM deposition. The native skin has a highly-complex microenvironment which comprises of hierarchical pore sizes (densely-packed matrix with small pores at the top papillary region to loosely-packed matrix with large pores at the bottom reticular region) [9]. As such, an optimal design for the dermal constructs should consist of a tri-zone (small pore size at top region to moderate pore size at middle region to large pore size at bottom region).

4.6. Design and develop bioprinting strategy for fabrication of optimal dermal skin constructs

The experimental results in earlier sections have highlighted the synergistic effect of MMC on 3D collagen-fibroblast matrices and importance of hierarchical porous structures in skin dermal constructs. A 3D porous scaffold offers a conducive microenvironment for the living cells to remodel and organize into a functional tissue [206]. The pores of the porous 3D scaffolds provide essential cues that regulate the cellular behaviour and function [207]. Notably, highly-complex hierarchical porous structures are commonly found in most biological tissues such as skin [9, 11], cornea [208] and even bone [209]. The importance of such hierarchical porous structures in native tissues has been critically reviewed [210] and it would be of utmost interest to recreate such complex hierarchical porous structures for tissue engineering applications. To date, fabrication of such hierarchical porous structures found in the native tissues remains highly challenging [211]. Some of the conventional approaches for fabrication of 3D porous scaffolds such as freeze drying, solvent casting-particulate leaching, gas foaming, phase separation, electrospinning have limited control and consistency over the scaffold architecture [201]. However, these fabrication processes can be detrimental to living cells and cell seeding is usually performed on the pre-fabricated scaffolds. Hence, there is a need for an alternative biocompatible fabrication strategy that offers good control over the pore sizes within a 3D matrix at high consistency.

3D bioprinting enables synchronized printing of multiple cells and materials to fabricate biomimetic 3D tissue-engineered constructs. Particularly, drop-on-demand (DOD) bioprinting offers a highly-versatile automated platform that precisely deposit nano-liter sized droplets of desired materials at pre-defined positions to create complex heterogeneous 3D collagen-based constructs. Recently, macromolecular crowding (MMC) is shown to influence collagen fibrillogenesis process [175-177]. The presence of the macromolecules alters the fibrillogenesis process via excluded volume effect and a printable macromolecular-based bio-ink is employed

to tune the porosity within a 3D-printed collagen-based matrix via DOD bioprinting. An ideal macromolecule-based bio-ink should have low viscosity ($\sim$ mPa.s range) [181] and high fractional volume occupancy (FVO) for enhanced excluded volume effect [177]. Among the various reported macromolecules, polyvinylpyrrolidone 360kDa (PVP) has been employed to enhance ECM deposition and cell proliferation in a dose-dependent manner [178] and also improve the homogeneity and viability of printed cells during/after bioprinting [190]. It exhibits a much higher FVO and lower viscosity as compared to previously studied macromolecules [178]. Here, a new printing approach termed as bioprinting-macromolecular crowding process (BMCP) facilitated the deposition of nano-litre sized droplets of collagen precursor, cross-linker and PVP macromolecules at pre-defined positions to manipulate the pore sizes within each printed collagen layer.

4.6.1. Development and optimization of PVP-based bio-inks

Different concentration of PVP-based bioinks (0-3% w/v) are prepared by mechanical agitation of the PVP powder (MW = 360 kDa, Sigma-Aldrich, St. Louis, MO, USA) in 1x PBS solution for 30 minutes. The rheology measurements of the PVP-based bio-inks are performed using a Discovery hybrid rheometer (TA instruments, USA). The rheology tests are measured within a linear viscoelastic region; the viscosities of PVP-based bio-inks are measured within the shear rates ranging from 10 to 1000 s^{-1} at a fixed temperature of 28 $^{\circ}\text{C}$ (same temperature as the printing chamber). A 3D bioprinter (RegenHU Biofactory®, Switzerland) with multiple microvalve-based print-heads of 100 μm nozzle diameter is used to evaluate the printability of PVP-based bio-inks. 15 arrays of 3x3 PVP-based droplets ($n=135$) are deposited at a fixed printing pressure of 0.25 bars onto 35 mm X 10 mm culture dishes (Corning®) (Fig 4-8).

Fig 4-8. (A) Schematic drawing of DOD bioprinting. (B) Rheological characterization of PVP-based bio-inks. (C) Representative images of printed PVP droplets (0-3% w/v); scale bar: 200 μm

The PVP-based bio-inks in this study have to satisfy the stringent printability requirements (eg. printable viscosity, printing stability and resolution). Rheological characterization is performed for all the PVP-based bio-inks (0-3% w/v) within the linear viscoelastic region; the PVP-based bio-ink exhibits Newtonian behavior as shown in Fig 4-8 (viscosity is independent of increasing shear rates) and the fluid viscosity generally increases with increasing PVP concentration (from ~ 0.84 mPa.s in 0% w/v PVP-based bio-ink to ~ 12.43 mPa.s in 3% w/v PVP-based bio-ink). Next, 15 arrays of 3x3 PVP-based droplets ($n=135$) are directly deposited into the culture dishes to evaluate the bio-ink printability. Good printability is observed for 0-2% PVP-based bio-inks (depicted by deposition of discrete 3x3 droplets of ~ 350 μ m diameter), whereas poor printability is observed for 3% PVP-based bio-ink (due to formation of fluid meniscus at the nozzle orifice even with increasing printing pressure). The optimal PVP-based bio-ink (highest polymer concentration that exhibits good printability) is selected for subsequent experiments. Hence, the 2% PVP-based bio-ink (highest printable concentration for maximum excluded volume effect) is selected for subsequent experiments.

Next, a study on the effect of valve opening time (VOT) on droplet resolution and accuracy is performed as the valve opening time of the microvalve-based printhead plays an influential role in affecting the droplet printing (Fig 4-9). The influence of VOT on the printing resolution and accuracy (deviation from the pre-defined position) is evaluated using the 2 % w/v PVP bio-ink. The printing resolution generally improves with decreasing VOT (from 558.3 ± 22.0 μ m at 0.5 ms to 314.0 ± 4.9 μ m at 0.1 ms). Further analysis on the deviation of the printed droplets away from the centre of pre-defined positions shows that the deviation increases with increasing VOT (from 17.2 ± 4.9 μ m at 0.1 ms to 44.4 ± 18.3 μ m at 0.5 ms). To ensure a fairer comparison between the different sample groups, printing accuracy (deviation distance from pre-defined positions as a percentage of droplet resolution) is performed on all the printed droplets. The printing accuracy generally improves with lower VOT (from 92.0 ± 3.3 % at 0.5 ms to $94.5 \pm$

1.6 % at 0.1 ms). In this work, a VOT of 0.1 ms was applied to achieve PVP printing at a printing resolution of $314.0 \pm 4.9 \mu\text{m}$ and printing accuracy of $94.5 \pm 1.6 \%$).

Fig 4-9. (A) Schematic drawing of DOD printing of PVP droplets, arrays of 3x3 PVP droplets are printed at a constant spacing of 600 μm . (B) Dispensing process of microvalve-based printhead is mainly controlled by the valve opening time (VOT). (C) Representative images of PVP droplets at varying VOT; scale bar: 200 μm (from left to right: 0.1 ms, 0.3 ms and 0.5 ms respectively) (D) Analysis of the printing resolution and accuracy (%) at varying VOT (0.1 ms – 0.5 ms).

4.6.2. Optimization of collagen-PVP printing process

To understand the influence of MMC on collagen formation kinetics, the collagen-fibroblast solutions are prepared at varying FVOs (0 – 54% whereby 0% FVO = 0% w/v PVP, 18% FVO = 0.4% w/v PVP, 36% FVO = 0.8% w/v, 54% FVO = 1.2% w/v) using different PVP concentrations and transferred to a 96-well plate (100 μ l per well). The collagen assembly is monitored at 28 $^{\circ}$ C by a time-lapse measurement of the light absorbance at 313 nm using a Tecan Infinite M200 spectrophotometer. The drop-on-demand printing process facilitates the deposition of different materials (collagen precursor, cross-linker, PVP-based bio-ink) as discrete droplets at pre-defined positions. Optimization of the printing speed, v , (feed rate: 200, 400, 600, 800 and 1000 mm/min) for collagen deposition is conducted to facilitate high-throughput printing of a thin and uniform circular collagen layer of 2 cm diameter. The printing time and thickness of each printed collagen layer are measured. The cross-linker solution (0.8 M sodium bicarbonate, NaHCO_3 , Sigma-Aldrich) is used to neutralize the acidic collagen precursor. Different droplet spacings, d , (1 mm, 2 mm, 3 mm, 4 mm) are used to determine the optimal spacing between the NaHCO_3 droplets for collagen cross-linking.

The rate of collagen I fibril formation is quantified using a turbidity test at an absorbance of 313 nm over a period of 30 minutes at every 1 minute interval at a constant temperature of 28 $^{\circ}$ C (temperature in the printing chamber) (Fig 4-10A). The collagen assembly can be determined via turbidity measurement as the assembled collagen fibers absorb light at 313 nm. The collagen nucleation (lag phase) is indicated by the 313nm absorbance at a value close to zero, following which the absorbance increases significantly during the fibrillogenesis (growth phase) until it reaches a final plateau which represented the complete fiber assembly. Under all crowding conditions, a typical sigmoidal curve is observed with a nucleation phase having absorbance values close to zero, a fibrillogenesis phase and a final plateau corresponding to the complete fiber assembly. The experimental results highlight the duration required for collagen

fiber assembly under different MMC conditions during the printing process; accelerated fiber assembly is observed within increasing PVP concentration.

Drop-on-demand bioprinting of collagen precursor and its crosslinking agent are performed using a RegenHU Biofactory® with multiple microvalve-based print-heads. The printed collagen precursor layer has to be adequately crosslinked with an alkaline solution to facilitate pH-dependent collagen cross-linking process. A mild alkaline buffer solution, sodium bicarbonate (NaHCO_3 , 0.8 M) is used in this work to facilitate homogeneous crosslinking of individual collagen layer on the 35mm X 10mm culture dishes (Corning®). Discrete NaHCO_3 droplets are printed at different droplet spacing, d , (1, 2, 3, 4 mm) directly below and above each printed collagen layer (NaHCO_3 -collagen- NaHCO_3). A droplet spacing of 2 mm results in optimal collagen cross-linking; a layer of cross-linked collagen hydrogel could be observed immediately after printing (stable collagen layer even when inverted). A droplet spacing of 1 mm results in excessive cross-linking of the collagen layer (a stable cross-linked collagen layer with excessive free-flowing NaHCO_3 buffer solution), whereas a droplet spacing of ≥ 3 mm crosslinking results in poor collagen cross-linking (as indicated by free-flowing collagen solution within the tilted culture dishes). The discrete NaHCO_3 droplets, that are deposited homogeneously below and above each printed collagen layer, facilitate complete crosslinking of the thin collagen layer over time and also serve as binding agent between the two adjacent printed collagen layers (Fig 4-10B). Hence, no delamination is observed between each printed collagen layer.

Fig 4-10. (A) Turbidity measurement of collagen kinetics formation at varying PVP concentration (0 – 1.2 % w/v PVP) at 28 °C. (B) Schematic drawing depicting the collagen crosslinking and bonding between adjacent collagen layers.

Next, optimization of the collagen printing parameters is conducted to achieve thin and homogeneous collagen layer at high-throughput rates (Fig 4-11). Discrete collagen droplets are deposited along adjacent lines (at a fixed spacing of 0.8 mm spacing) at different feed rates, v , (200, 400, 600, 800 and 1000 mm/min). Generally, increasing feed rates shorten the printing time for each printed collagen layer (from ~127 seconds at 200 mm/min to ~33 seconds at 1000 mm/min) and reduce the thickness of each printed collagen layer (from $75.0 \pm 3.8 \mu\text{m}$ at 200 mm/min to $19.8 \pm 3.3 \mu\text{m}$ at 1000 mm/min). The feed rate of 1000 mm/min results in gaps within the circular 2cm diameter collagen layer. Hence, an optimal feed rate of 800 mm/min is selected for subsequent collagen printing ($\sim 22.5 \pm 3.1 \mu\text{m}$ collagen thickness within 40 seconds). The optimal printing parameters for printing of collagen layers are at a constant NaHCO₃ droplet spacing of 2 mm and a feed rate of 800 mm/min for collagen printing. This

facilitates the printing of thin and homogeneously cross-linked collagen layers [182] (circular layer of 2 cm diameter) at a high-throughput rate ($22.5 \pm 3.1 \mu\text{m}$ collagen thickness within 40 seconds).

Fig 4-11. Influence of printing feed rates on collagen printing time and thickness

4.6.3. Fabrication and characterization of hierarchical porous collagen-based constructs

Using the optimized printing parameters for collagen printing (droplet spacing of 2 mm and feed rate of 800 mm/minute), samples of 6-layered hierarchical collagen structures ($n=12$) are printed using a facile single-step bioprinting strategy. Droplets of cross-linking solution (NaHCO_3) are first deposited prior to the deposition of subsequent collagen layer. Next, discrete droplets of PVP-based bio-ink are then deposited over the cross-linked collagen layer at a droplet spacing of 1mm. The PVP concentration within each printed collagen layer is controlled by depositing different number of PVP droplets at each pre-defined position (1, 2 or 3 droplets per shot) to manipulate the porosity of each printed collagen layer. The cross-linking solution (NaHCO_3) is then deposited over the collagen-PVP layer to fully cross-link the collagen layer. The process (NaHCO_3 -Collagen-PVP- NaHCO_3) is then repeated (from bottom to top) to fabricate a multi-layered construct in a layer-by-layer printing process. The final printed constructs are then incubated at 37 °C overnight in culture medium before performing Field Emission Scanning Electron Microscope (FE-SEM). The printed samples are first dehydrated using graded ethanol solutions (25%, 50%, 75%, 95% and lastly 100% ethanol for 20 minutes each). Next, the samples are placed in a critical point dryer from Leica EM CPD030, Germany and flushed with cold liquid CO_2 at 4 °C over a period of 2 hours to preserve the nanostructure within the printed collagen matrices. The dried samples are then carefully sectioned using sterile surgical blade to expose the cross-sectional area of the hierarchical porous collagen-based construct. The cross-sectional area of the collagen specimens are mounted onto aluminium stubs and coated with platinum (Pt) using Polarin SC7640 Sputter Coater from Quorum Technologies, United Kingdom. The cross-sections are examined with Ultra-Plus Field Emission Scanning Electron Microscope (FE-SEM) from Carl Zeiss, Germany. The electron images are taken at 2 keV accelerating voltage. Lastly, ImageJ analysis are performed on FE-SEM images ($n=10$) to evaluate the pore size and porosity of the bioprinted hierarchical constructs.

A new fabrication strategy is proposed in this work (Fig 4-12); this Bioprinting-Macromolecular Crowding Process (BMCP) facilitates drop-on-demand bioprinting of discrete macromolecule-based bio-ink directly over each individual printed collagen layer to tune the collagen fibrillogenesis process. The presence of macromolecules within the collagen matrix accelerates the collagen fibrillogenesis process and tunes the internal architecture of the collagen matrix. An increasing PVP concentration accelerates the collagen fibrillogenesis process and increases the pore size of the collagen matrix. Using a single print-head containing the 2% w/v PVP-based bioink, the PVP concentration can be controlled by varying the number of PVP droplets per shot (no. of printed droplets during each valve opening time) at pre-defined positions as indicated by the red dots in Step 3 of Fig 4-12. As a proof-of-concept, hierarchical porous collagen structures consisting of 3 different regions (top, middle and bottom regions) are printed. The number of PVP droplets per shot within each printed region is varied (top region: 1 PVP droplet – 0.4 % w/v PVP, middle region: 2 PVP droplets – 0.8 % w/v PVP, bottom region: 3 PVP droplets – 1.2 % w/v PVP) to fabricate hierarchical porous collagen-based structures. The PVP w/v % within each printed layer is calculated based on the volume ratio between the cross-linked collagen layer and PVP droplets. As shown in Fig 4-12 (Bottom), the PVP droplets can be visualized as tiny spheres with hydrodynamic radii exerting excluded volume effect (EVE) on the surrounding collagen molecules. This macromolecular crowding hinders solute diffusion and hence accelerates the collagen fibrillogenesis process (rate of fiber formation increases with increasing PVP concentration). Furthermore, increasing number of PVP droplets result in a higher macromolecular concentration which induces the formation of larger pores.

Fig 4-12. (Top) Schematic drawing of Bioprinting-Macromolecular Crowding Process (BMCP); a 6-layered hierarchical collagen construct was printed in a bottom-up layer-by-layer fabrication approach. The architecture within each printed collagen layer is manipulated by the number of PVP droplets per shot (no. of printed droplets during each valve opening time) at pre-defined positions as indicated by red dots in Step 3 of BMCP. (Bottom) Influence of MMC on collagen architecture; increasing PVP droplets exerting a more significant EVE on surrounding collagen fibrils.

The representative FE-SEM image in Fig 4-13 illustrates a hierarchical porous structure that varies in its pore size and porosity across the thickness of the entire construct. Further magnification of the hierarchical porous collagen constructs reveals distinct differences in the architecture of the 3 different regions. ImageJ analysis of the collagen architecture at the top region shows a low porosity of 7.4% with an average pore size of $0.50 \pm 0.13 \mu\text{m}$, whereas the collagen architecture of the bottom region shows a higher porosity of 46.7% with an average pore size of $1.62 \pm 0.51 \mu\text{m}$. A significant difference in the pore size across the different regions is observed. Generally, the results indicate that the pore size within each printed region increases with increasing PVP droplets.

Fig 4-13. (A) FE-SEM images of representative bioprinted hierarchical porous collagen-based structures; analysis of (B) pore size and (C) porosity of bioprinted collagen structures with the 3 regions.

The pre-defined hierarchical configuration of the printed collagen constructs (3 different regions of equal thickness – top, middle and bottom) is enclosed by the blue dotted lines (Fig 4-14). Due to the differences in PVP concentration across the 3 printed regions, it is highly possible that the PVP macromolecules diffused from bottom (high PVP concentration) to top (low PVP concentration). The printed 6-layered constructs have an average thickness of $156.5 \pm 5.7 \mu\text{m}$ and two different transition zones (marked by red dotted lines) are observed. A transition zone of $11.2 \pm 2.1 \mu\text{m}$ between bottom-middle region and a transition zone of $6.7 \pm 1.9 \mu\text{m}$ between middle-top region. A higher PVP concentration (1.2 % w/v) resulted in a higher diffusion flux at the bottom-middle region (formation of a wider transition zone) as compared to middle-top region. Although it is challenging to restrict the macromolecule diffusion across the different regions, this bioprinting strategy facilitated the fabrication of hierarchical porous structures by manipulating the PVP concentration at different regions.

Fig 4-14. Printing accuracy of the pre-defined hierarchical configuration; scale bar: 20 μm

4.6.4. Cell proliferation and viability in printed collagen-fibroblast constructs

To further demonstrate the feasibility of incorporating living cells in the BMCP, an additional microvalve-based print-head containing primary normal human dermal fibroblasts (Promocell, Germany) is used in the printing process. The additional printing cartridge consisting of 1 million fibroblasts/ml in PVP-based bio-ink is used to print discrete cell droplets onto each printed collagen layer. The printed constructs (consisting of 6-layered structure, $n=18$) are then incubated over a period of 10 days to evaluate the cell viability and proliferation rate. Prestoblu[®] assay (Thermo Fisher Scientific, according to manufacturer's protocol) is used to determine the cell viability and proliferation rate ($n=3$). Furthermore, 3 random samples are stained with Molecular Probes[®] Live/Dead staining kits (Thermo Fisher Scientific, according to manufacturer's protocol) to evaluate the cell morphology and spreading at different time intervals (Day 3, Day 7, Day 10). It is to be noted that there is a presence of both "focused" and "unfocused" cells on the same plane captured in the microscopic images as the cells are deposited at all 6 different layers (Fig 4-15).

The increase in the number of living cells (represented by green in Fig 4-15A) over a period of 10 days has indicated that the BMCP is biocompatible and does not exert detrimental effect on the printed cells. Quantitative analysis is also conducted using the PrestoBlue[®] assay, the measured relative fluorescence units (RFUs) is directly proportional to the number of living cells and all the measured values are normalized to Day 3 for easy comparison. The normalized RFUs over a period of 10 days have confirmed that the primary cells are continually proliferating within the printed collagen constructs. Furthermore, ImageJ analysis of the stained living cells (cell perimeter and cell area) indicates that the elongated fibroblasts are gradually spreading within the collagen matrix (as highlighted by the increase in both cell perimeter and cell area over time in Fig. 4-15). Therefore, the proposed BMP can be utilized for fabrication of hierarchical structures containing living cells.

Fig 4-15. (A) Representative images of printed primary normal human dermal fibroblasts within PVP-collagen matrix at different time intervals (Day 3, 7 and 10), scale bar: 200 μm . (B) Analysis of cell proliferation using normalized relative fluorescence units (RFUs) from PrestoBlue® assay at different time intervals (Day 3, 7 and 10). Analysis of cell spreading by evaluating (C) cell perimeter and (D) cell area at different time intervals (Day 3, 7 and 10).

4.7. Discussion

This study investigates the synergistic effect of MMC on 3D collagen-fibroblast matrices; the experimental data indicates that MMC has significant effect on both the cell-matrix remodeling process and cellular behavior. The results also highlight the importance of hierarchical pore sizes within the 3D collagen-fibroblast matrices. The 3D matrices of 0 % FVO (densely-packed matrix with small pore sizes) enhance cell growth but hinder ECM deposition, the 3D matrices of 18 – 36 % FVO (matrix with moderate pore sizes) experience minimal matrix contraction and exhibit moderate cell growth and ECM deposition, lastly the 3D matrices of 54 % FVO (loosely-packed matrix with large pore sizes) demonstrate slow cell growth but significantly higher ECM deposition. As such, an optimal design for the dermal skin constructs should consist of a tri-zone (small pore size at top region to moderate pore size at middle region to large pore size at bottom region).

The new bioprinting strategy has demonstrated that collagen architecture (pore size and porosity) within each printed layer can be manipulated by varying the PVP concentration in the proposed Bioprinting-Macromolecular Crowding Process (BMCP). The uniform deposition of discrete droplets of cross-linkers (NaHCO_3) and macromolecules (PVP) over each thin printed collagen layer ($\sim 20 \mu\text{m}$) facilitates the rapid and homogeneous cross-linking. The presence of PVP macromolecules not only accelerates the collagen fibrillogenesis process but also tunes the collagen architecture in a controlled manner. The PVP macromolecules exert an excluded volume effect (which is dependent on both electrostatic repulsions and non-specific steric hindrances) on the surrounding collagen molecules; an increasing PVP concentration results in formation of larger pores and increases porosity within the 3D collagen matrices. This new printing strategy facilitates the fabrication of hierarchical porous collagen-based constructs via drop-on-demand bioprinting process in a highly-controlled manner.

5. Proof-of-Concept: 3D Bioprinting of Pigmented Human Skin Constructs

5.1. Development of pigmented human skin constructs

Most of the prior studies on skin bioprinting focus mainly on the use of keratinocytes (KCs) and fibroblasts (FBs) to fabricate 3D skin constructs. The melanocyte, a critical component in the epidermal melanin units [6], is critical for investigation of skin pigmentation in an *in-vitro* 3D physiological model. Furthermore, the incorporation of melanocytes (MCs) to the current 3D bioprinted skin constructs would enable the development of pigmented skin models for potential cosmetics and toxicology testing. A prior study has demonstrated that the transplantation of skin equivalents consisting of melanocytes from Chinese donors (pale pigmentation) on the nude mice resulted in the formation of black-pigmented skin [7]. In a recent study, conventional fabrication of 3D pigmented skin models was demonstrated by using an optimized co-culture medium (consisting of various growth factors and pro-pigmenting agents) [8], however uneven pigmentation was observed. The bioprinting of 3D pigmented human skin constructs will provide a highly relevant approach to investigate the intricate role of various cell-cell, cell-matrix interactions in the regulation of skin pigmentation. As a proof-of-concept, this work demonstrates the feasibility of fabricating 3D pigmented human skin constructs by combining bioprinting approaches with other strategies such as macromolecular crowding and co-culture techniques.

5.2. Preparatory Work

5.2.1. Formulation of co-culture medium for different skin cells

The lack of suitable medium to support the co-culture of all three cell types is hindering the fabrication of pigmented skin models. Prior to the bioprinting process, optimization of the co-culture medium is performed by culturing the three different types of skin cells individually in different mixture of culture medium (KGM, MGM and FGM). The optimal co-culture medium should support the growth of all three types of primary skin cells. In this work, different co-culture media (KGM, MGM, FGM) are mixed in different ratio to formulate an optimal co-culture medium. As a control, recommended culture medium is also used for the co-culture. It is difficult to culture three different types of skin cells using a single type of control medium over a prolonged duration. The experimental results for KGM:MGM (2:1) and KGM:MGM (3:1) are presented and benchmarked against the control medium (KGM, MGM and FGM for the respective cell type). To develop a co-culture medium, the influence of the co-culture medium on the cell proliferation rate is analyzed. The cell proliferation rate over a period of 7 days is used as a fundamental indicator to determine an optimal co-culture medium.

Among the three types of skin cells, primary adult human fibroblasts are the most adaptable and the fibroblasts can continue to grow and proliferate in other control medium (KGM and MGM) but at a slower rate (as compared to FGM in Fig 5-1). Generally, as fibroblast growth rate decreases as the ratio of KGM:MGM increases. Next, further observations revealed that the keratinocytes are highly susceptible to the change in culture medium. A high KGM:MGM ratio of at least 3:1 is required to ensure that the keratinocytes continue to proliferate over time (Fig 5-2). Lastly, the melanocytes are observed to exhibit slower growth rate as the ratio of KGM:MGM increases (Fig 5-3). As the objective of this study is to develop a co-culture medium for the fabrication of pigmented skin models, an optimal co-culture medium of KGM:MGM (3:1) is selected (without any detrimental effect on each type of skin cells).

Fig 5-1. (A) Representative bright-field images of fibroblasts cultured in different culture medium (control medium, KGM:MGM (2:1) and KGM:MGM (3:1)). (B) Influence of various co-culture medium (control medium, KGM:MGM (2:1) and KGM:MGM (3:1)) on fibroblast proliferation rate; scale bar: 200 μ m. Significance levels are as follows: $p < 0.001$ (***), $p < 0.01$ (*).

Fig 5-2. (A) Representative bright-field images of keratinocytes cultured in different culture medium (control medium, KGM:MGM (2:1) and KGM:MGM (3:1)). (B) Influence of various co-culture medium (control medium, KGM:MGM (2:1) and KGM:MGM (3:1)) on keratinocyte proliferation rate; scale bar: 200 μ m. Significance levels are as follows: $p < 0.001$ (***), $p < 0.01$ (*).

Fig 5-3. (A) Representative bright-field images of melanocytes cultured in different culture medium (control medium, KGM:MGM (2:1) and KGM:MGM (3:1)). (B) Influence of various co-culture medium (control medium, KGM:MGM (2:1) and KGM:MGM (3:1)) on melanocyte proliferation rate; scale bar: 200 µm. Significance levels are as follows: $p < 0.001$ (***), $p < 0.01$ (*).

5.2.2. Influence of cell density on epidermal thickness

A critical aspect to achieving skin pigmentation is associated with the transfer of melanosomes from the melanocytes to the surrounding keratinocytes in the epidermal region. It was reported in a study that the melanosome uptake is highly dependent on the keratinocyte proliferation and differentiation [212]; the melanosome uptake is significantly higher in the differentiation keratinocytes as compared to the proliferative keratinocytes. As such, it is important to provide an optimal keratinocyte density for proliferation and differentiation into a well-stratified epidermal region. Different keratinocyte densities (low cell density - 50,000 keratinocytes/cm², medium cell density - 75,000 keratinocytes/cm², high cell density - 125,000 keratinocytes/cm²) are directly printed over the dermal skin region (3D collagen-fibroblast matrices) to evaluate the influence of keratinocyte density on epidermal thickness after 2 weeks of air-liquid interface (ALI) culture.

A low cell density (50,000 keratinocytes/cm²) results in the formation of a relative thin epidermal region (26.8 ± 2.4 µm thickness), a medium cell density (75,000 keratinocytes/cm²) results in the formation of a thicker epidermal region (54.2 ± 4.7 µm thickness) and lastly a high cell density (125,000 keratinocytes/cm²) results in the formation of the thickest epidermal region (72.2 ± 3.7 µm thickness) after 2 weeks of ALI culture. Generally, the addition of more keratinocytes (from 75,000 keratinocytes/cm² to 125,000 keratinocytes/cm²) results in the formation of thicker epidermal region as shown in Fig 5-4 (Top). However, it is important to note that a further increase in keratinocyte density has diminishing effect on the increase of epidermal thickness, as indicated by Fig 5-4 (Bottom). Hence, a suitable keratinocyte density of 125,000 keratinocytes/cm² is selected for subsequent experiments.

Fig 5-4. (Top) Representative images of epidermal thickness at varying keratinocyte density after 2 weeks; scale bar: 50 μm . (Bottom) Analysis of influence of keratinocyte density on epidermal thickness.

5.2.3. Influence of air-liquid interface (ALI) duration on epidermal thickness

Another important parameter is the influence of ALI culture period on the epidermal thickness. As discussed in the earlier sections, the melanosome uptake is highly dependent on the degree of keratinocyte differentiation. The ALI culture is a critical process known to induce the keratinocyte differentiation for formation of well-stratified keratinocyte layers [213]. The average thickness of the epidermal skin region varies among the different anatomical parts of the human body, it typically measures around 150 – 200 μm in thickness [12]. A cell density of 125,000 keratinocytes/ cm^2 is directly printed over the dermal skin region (3D collagen-fibroblast matrices) to evaluate the influence of ALI duration (1 week, 2 weeks and 4 weeks) on epidermal thickness.

A longer ALI culture generally results in the formation of thicker epidermal region (enclosed by the dotted lines – Fig 5-5). The thickness of the epidermal region increases from $51.6 \pm 3.4 \mu\text{m}$ to $76.3 \pm 4.2 \mu\text{m}$ to $143.5 \pm 6.7 \mu\text{m}$ after 1 week, 2 weeks and 4 weeks of ALI culture respectively. The proliferative keratinocytes at the basal layers of the native human skin are known to undergo a sequential differentiation process within the epidermal region over a period of 21 -28 days [9]. Furthermore, the presence of flattened cell morphology within the epidermal region after 2 weeks of ALI culture indicates early signs of keratinocyte differentiation, which is critical for the melanosome uptake. Unlike the native human skin, it is also challenging to culture the *in-vitro* skin constructs over pro-longed duration due to cellular senescence. Hence, an ALI culture period of 4 weeks is selected because of the formation of well-stratified keratinocyte layers with relatively similar epidermal thickness ($143.5 \pm 6.7 \mu\text{m}$) to native epidermal skin thickness.

Fig 5-5. (Top) Representative images of epidermal thickness at different ALI culture period; scale bar: 50 μm. (Bottom) Analysis of influence of ALI culture period on epidermal thickness.

5.3. Fabrication of 3D pigmented human skin constructs

A two-step bioprinting strategy comprising of the fabrication of a biomimetic dermal skin construct comprising of hierarchical porous collagen-based constructs and patterning of the epidermal cells (KCs and MCs) over the biomimetic dermal constructs is implemented (Fig 5-6). The fabrication of hierarchical porous collagen-based constructs and the optimization of DOD bioprinting of cell droplets (KCs and MCs) are reported previously in the earlier chapters. The 3D bioprinted constructs are fabricated using RegenHU Biofactory® with multiple microvalve-based print-heads of 100 µm nozzle diameter in two separate steps: **Step 1.** 3D bioprinting of collagen-fibroblast matrices onto 6-well culture inserts, which are subsequently cultured over a period of 4 days, **Step 2.** KCs and MCs are directly printed onto the bioprinted collagen-fibroblast matrices. The epidermal cells (KCs and MCs) are patterned uniformly over the top surface of 3D bioprinted dermal constructs. Each melanocyte droplet is surrounded by 8 keratinocyte droplets (repeats of 3 x 3 array) and overlapping printing of cell droplets is performed to emulate the functional epidermal-melanin units [6].

The results on engineering 3D complex microstructure in collagen-fibroblast matrices in Chapter 4 have indicated that an optimal period of 4 days is necessary for ECM secretion prior to addition of epidermal skin cells on top of the dermal skin region. The different skin cells (keratinocytes and melanocytes) are printed using 2.5% w/v PVP-based bio-ink at a constant printing pressure of 0.25 bars through a 100 µm diameter nozzle orifice to improve the cell viability and homogeneity according to the results from development and optimization of PVP-based bio-ink in Chapter 3. This facilitates the patterning of skin cells with high viability with controlled cell density (**in preparatory work**). The 3D bioprinted constructs are cultured under submerged conditions using the optimal co-culture medium over a period of 7 days prior to tissue maturation at air-liquid interface (ALI) for a further 4 weeks [175] (**in preparatory work**).

Fig 5-6. (A) Schematic drawing for bioprinting strategies for design and fabrication of 3D pigmented human skin models. (B) Fabrication process for the 3D pigmented human skin constructs.

The conventional pigmented skin constructs are fabricated using the manual-casting approach (Fig 5-7). The collagen-fibroblast dermal constructs are fabricated by manually casting the cross-linked collagen hydrogel precursor into 6-well culture inserts. The dermal constructs are subsequently cultured over a period of 4 days using FGM prior to manual pipetting of KCs and MCs (final cell density of 125,000 KCs/cm² and 10,000 MCs/cm²) over the collagen-fibroblast matrices. The final manually-cast constructs are cultured under submerged conditions using the optimal co-culture medium over a period of 7 days. After which; the manually-cast constructs are subjected to tissue maturation at air-liquid interface (ALI) for a further 4 weeks using the optimal co-culture medium.

Fig 5-7. Fabrication process for the 3D manual-cast human skin constructs.

5.4. Characterization of 3D pigmented human skin constructs

5.4.1. Comparison of 3D bioprinting and manual-casting approaches

Both the 3D bioprinted pigmented human skin constructs and manually-cast pigmented human skin constructs are fabricated using same type of skin cells at similar cell density and culture conditions. Bright field images are first captured using a microscope to demonstrate the ability to control cell distribution using 3D bioprinting technique (Inverted Microscope system IX53, Olympus, Japan). Next, field-emission scanning electron microscopy (FE-SEM) is performed on the fabricated dermal constructs ($n = 3$) from both sample groups. The samples are first dehydrated using graded ethanol solutions. After which; the samples are placed in a critical point dryer from Leica EM CPD030, Germany and flushed with cold liquid CO₂ at 4 °C over a period of 2 hours to preserve the microstructure within the 3D collagen matrices. The dried samples are then carefully sectioned using sterile surgical blade to expose the cross-sectional area of the collagen-based constructs. The cross-sectional area of the specimens are mounted onto aluminium stubs and coated with platinum (Pt) using Polarin SC7640 Sputter Coater from Quorum Technologies, United Kingdom. The cross-sections are examined with Ultra-Plus Field Emission Scanning Electron Microscope (FE-SEM) from Carl Zeiss, Germany.

The two distinct differences between the two fabrication approaches (3D bioprinting and manual-casting) are the cell distribution on top of the dermal regions and the microstructures within the dermal regions (Fig 5-8). Random distribution of cells is observed in the manual casting approach, whereas the cells are deposited as cell droplets in a highly repeatable manner. Furthermore, it is highly challenging to tune the microstructure within the 3D collagen matrix using the manual-casting approach. In contrast, the use of bioprinting strategy can be used to tune microstructure of the 3D collagen matrix to achieve a hierarchical porous structure (Fig 5-8).

Fig 5-8. The implementation of 3D bioprinting strategy to control the cell distribution on dermal surface (cells indicated by black arrows) (scale bar: 100 μm) and microstructure within 3D collagen dermal matrix (scale bar: 20 μm) of the pigmented skin constructs.

The representative images of the pigmented skin models from both fabrication approaches after 4 weeks of ALI culture are shown in Fig 5-9. It is observed that top surface of the manually-cast construct exhibited a concave shape. This is likely due to significant contractile forces from epidermal cells (KCs and MCs) that are seeded on the surface of the dermal region. During the cell seeding process, to ensure that the epidermal cells are seeded directly on top surface of the dermal region instead of the underlying trans-well membrane, most of the cells are carefully pipetted near the center of the top surface. Hence, this could lead to non-homogeneous distribution of epidermal cells (KCs and MCs) over the dermal region (more cells clustered to the core of the top surface). Uneven skin pigmentation is observed on the manually-cast 3D pigmented skin constructs and the presence of dark-pigmented spots is clearly evident in Fig 5-9.

In contrast, the experimental results show uniform pigmentation on the 3D bioprinted pigmented human skin constructs and the resulting skin pigmentation is similar to that of the Caucasian donors with pale pigmentation. The results demonstrate uniform skin pigmentation on the 3D bioprinted pigmented human skin constructs (using three different skin cells from three different Caucasian donors with pale pigmentation).

Fig 5-9. Representative images of pigmented skin models fabricated via two different approaches. (Left) 3D-bioprinted pigmented human skin constructs with uniform skin pigmentation, (Right) Manually-cast skin constructs with uneven pigmentation (presence of dark-pigmented spots). Scale bar: 2 mm.

5.4.2. Histological analysis

To perform histological characterization, samples from both manual-casting and 3D bioprinting groups (n=6, for each group) are embedded in optimum cutting temperature (OCT) by freezing in a slurry of 2-methylbutane and dry ice prior to cryostat sectioning using a research cryostat (Leica CM3050 S) at – 22 °C. For haematoxylin and eosin (H&E) staining, the fresh frozen samples on the slides are first placed into fixative until the tissues are fully fixed. After which; the slides are dried in a desiccator for an additional 15 minutes before rinsing in water. The sections on the slides are immediately stained with H&E and dehydrated through graded ethanol solutions followed by a xylene-based mountant. For fontana masson (FM) staining, staining protocol is performed according to manufacturer's instructions (Catalogue no. ab150669, Abcam). Further analysis (H&E staining and FM staining) is conducted to characterize both pigmented skin models (the epidermal regions are enclosed by the dotted lines in Fig 5-10). In the manual casting approach, accumulation of granules is clearly evident in the stratified epidermal region. In contrast, a well-developed stratified keratinocyte layers (presence of cornified envelope near surface and gradual transition of rounded proliferative keratinocytes near the basal lamina to flattened differentiated keratinocytes near the cornified envelope) is observed in the 3D bioprinted skin constructs. The 3D bioprinted skin constructs show great morphological resemblance to the native skin tissue. In the subsequent FM staining (Fig 5-11), it is confirmed that there is accumulation of large melanin granules within epidermal region of the pigmented skin constructs fabricated by manual casting approach (as indicated by the presence of numerous dark-pigmented spots discussed in the previous section). On the other hand, small melanin granules are uniformly distributed across the epidermal region of the 3D bioprinted skin constructs (mostly found in the granular layer).

Fig 5-10. H&E staining of 3D pigmented human skin constructs. (Left) 3D bioprinting approach, (Right) Manual casting approach; scale bar: 50 μ m. The epidermal region is indicated by **E** and the dermal region is indicated by **D**.

Fig 5-11. FM staining of 3D pigmented human skin constructs. (Left) 3D bioprinting approach, (Right) Manual casting approach; scale bar: 50 μ m. Arrows indicating the distribution of melanin granules within the epidermal region of 3D matrices (stained brownish-black).

5.4.3. Immunochemical analysis

For immunofluorescence staining, the 10 μm cryosections are first fixed in a mixture of methanol and acetone (in a 1:1 ratio) at -20°C for 20 minutes, followed by air-drying in a fume hood. The dried samples are then washed with tris buffered saline (TBS) supplemented with 0.1 % triton-X (washed thrice, 5 minutes per wash at room temperature). Non-specific binding of antibodies is blocked using 10 % fetal bovine serum (heat inactivated) in tris buffered saline (TBS) supplemented with 0.1 % triton-X for an hour at room temperature and the samples are incubated with anti- collagen IV antibody (Abcam; dilution 1:100), anti-cytokeratin 1 antibody (Abcam; dilution 1:100), anti-cytokeratin 6 antibody (Abcam; dilution 1:100) and anti-melanoma antibody, HMB45 (Abcam; dilution 1:100) at 4°C overnight. The samples are then washed with tris-buffered saline (TBS) supplemented with 0.1 % triton-X (washed thrice, 5 minutes per wash at room temperature). Secondary antibody (Alexa Fluor 488 from Abcam; dilution 1:1000) is then added for an hour at room temperature. Cell nuclei are counterstained using VectaShield Hardset™ with DAPI. Samples are then left to dry for 24 hours prior to visualization using Olympus fluorescent microscope (Fig 5-12).

Fig 5-12. Immunofluorescence staining of (Top) 3D bioprinted pigmented human skin constructs and (Bottom) manually-cast pigmented skin constructs, counterstained with DAPI. Scale bar: 50 μm

The pigmented skin constructs are stained for the presence of collagen VII (Col VII, an important anchoring protein found in the basement membrane at the epidermal-dermal junction), HMB-45 (distribution of melanocytes within the epidermal region), keratin 1 (K1, indicator of differentiated keratinocytes) and keratin 6 (K6, indicator of proliferative keratinocytes). Positive staining for all the antibodies (col VII, HMB-45, K1 and K6) is observed in the 3D bioprinted skin constructs, whereas there is only positive staining for HMB-45 and K6 in the manually-cast skin constructs (Fig 5-12). The presence of collagen VII is critical for the attachment and positional orientation of melanocytes at the epidermal-dermal junction [15]. This is corroborated by another study which indicated the absence of basement membrane proteins (Col VII) in control manually-cast samples at week 4 [175]. Further observation for HMB-45 staining reveals there are more melanocytes found near the epidermal-dermal junction in the 3D bioprinted constructs as compared to the manually-cast constructs. This is due to the presence of the thin collagen VII layer at the epidermal-dermal junction which facilitates melanocyte attachment and proliferation. The presence of K1 layer on the 3D bioprinted constructs indicates the presence of differentiated keratinocytes on the outermost layer of the epidermal region (confirming the formation of well-developed stratified keratinocyte layers in the H&E staining of 3D bioprinted constructs). The presence of differentiated keratinocytes is essential for the melanin transfer (from melanocytes to keratinocytes) [107, 214]. The K6 staining also reveals a layer of highly proliferative keratinocyte cells at the basal layer of the epidermal region (eventually undergo sequential differentiation process to form high differentiated keratinocyte cells over time), whereas majority of the epidermal region of the manually-cast skin constructs is stained positively for K6 (absence of differentiated keratinocytes).

5.5. Discussion

As a proof-of-concept, this work demonstrates the feasibility of applying a two-step bioprinting-based strategy to fabricate 3D pigmented human skin constructs (using 3 different skin cells from 3 different skin donors). A suitable co-culture medium of KGM:MGM (3:1) is formulated to support and maintain the growth of all 3 types of skin cells. The bioprinting strategy facilitates the deposition of cell droplets to emulate the epidermal melanin units (pre-defined patterning of keratinocytes and melanocytes at the desired positions) and manipulation of 3D microenvironment to fabricate 3D biomimetic hierarchical porous structures. The ability to control the spatial arrangement of the keratinocytes and melanocytes in highly-specific manner and to fabricate biomimetic 3D hierarchical porous structures facilitate critical cell-cell and cell-matrix interactions that enable the fabrication of 3D bioprinted pigmented human skin constructs with uniform skin pigmentation. The histological analysis indicates the presence of a well-developed epidermal region and uniform distribution of melanin granules in epidermal region of the 3D bioprinted pigmented skin constructs. Furthermore, the immunochemical analysis reveals that the presence of important biomarkers (col VII, HMB-45, K1 and K6) in the 3D bioprinted pigmented skin constructs, whereas there is only presence of HMB-45 and K6 biomarkers in the manually-cast constructs. Although 3D bioprinting is an advanced manufacturing platform, the incorporation of other strategies such as macromolecular crowding and co-culture techniques are also critical toward the fabrication of 3D pigmented human skin constructs.

6. Conclusions and Future Work

6.1. Conclusions

6.1.1. Developing suitable bio-inks for improved cell viability and homogeneity

The bioprinting of living cells is an intricate printing process; the two critical stages of bioprinting include the high shear stress within the nozzle orifice and the droplet impact upon hitting the substrate surface. The bio-ink properties play a critical role in the cell viability and homogeneity during the printing process. The incorporation of PVP molecules within the bio-inks induces a change in the Z value (viscosity, surface and density) and affects the bio-ink printability. In this work, the printability of the bio-inks is determined to be within a range of Z values ($5.54 \leq Z \leq 64.36$). A bio-ink with higher PVP concentration (lower Z value) results in slower droplet velocity and offers extra cushioning effect (higher energy dissipation) for the printed cells to increase the cell viability during the printing process. The results also indicate that a change in cell concentration (from 0.5 mil cells/ml to 2.0 mil cells/ml) has no significant effect on the Z values and its corresponding cell viability. The printed cells that are cultured over a period of 96-hours, do not exhibit significant printing-induced damage when the Z values of the PVP-based bio-inks are below 9.30. Furthermore, a higher PVP concentration also alleviates the issue of cell sedimentation and adhesion inside the printing cartridge during the printing process. This study elucidates the effect of bio-ink properties on viability and homogeneity of printed cells during the DOD bioprinting process and provides essential information for the development of new printable bio-inks for higher cell viability and homogeneity.

6.1.2. Engineering the complex 3D microenvironment in collagen-fibroblast matrices

In this study, the influence of MMC (0 – 54 % FVO) on cell-matrix remodeling process and cellular behavior is investigated. Although the MMC is known to have significant effect on living cells in 2D culture system and modulation of 3D microenvironment, little information is known about the synergistic effect of MMC on both living cells and 3D microenvironment of collagen-based matrix. Different 3D collagen hydrogels with different fibrillary architecture are fabricated by supplementing the pre-fibrillated collagen solution (constant concentration of 3.34 mg/mL) with varying PVP concentration to achieve the desired FVO (0 – 54 %). The MMC induces a significant change on the pore size of 3D microenvironment (larger pore size with increasing FVO) and it plays an influential role in regulating cellular behavior such as matrix contraction, cell-matrix remodeling process and lastly ECM secretion. The study highlights the importance of hierarchical pore sizes within the 3D dermal skin constructs and provides critical insights (optimal pore size range for each region of the proposed tri-zone dermal constructs and optimal incubation duration) for the development of improved dermal constructs using MMC. The experimental results have highlighted the importance of a biomimetic hierarchical porous structure in emulating native skin. The 3D collagen architecture (pore size and porosity) within each printed layer can be manipulated by varying the PVP concentration in the new bioprinting strategy known as Bioprinting-Macromolecular Crowding Process (BMCP). The uniform deposition of discrete droplets of cross-linkers (NaHCO_3) and macromolecules (PVP) over each thin printed collagen layer (~20 μm) facilitates the rapid and homogeneous cross-linking. The presence of PVP macromolecules not only accelerates the collagen fibrillogenesis process but also tunes the collagen architecture in a controlled manner. The PVP macromolecules exert excluded volume effect (which is dependent on both electrostatic repulsions and non-specific steric hindrances) on the surrounding collagen molecules; an increasing PVP concentration results in formation of larger pores and increases porosity within the 3D collagen matrices.

6.1.3. Proof-of-concept: bioprinting of 3D pigmented human skin constructs

The development of 3D pigmented skin constructs therefore presents a highly relevant approach to investigate the intricate role of various cell-cell, cell-matrix and epithelial-mesenchymal interactions in the regulation of skin pigmentation. This work demonstrates the feasibility of applying bioprinting-based strategies to achieve uniform pigmentation with artificial human skin (using 3 different skin cells from 3 different skin donors). A two-step bioprinting strategy is implemented to demonstrate the feasibility of fabricating 3D pigmented human skin constructs. The bioprinting strategy involves the bioprinting of biomimetic, hierarchical porous dermal constructs and patterning of EMUs. The 3D bioprinted constructs show significant improvement in term of uniform pigmentation as compared to the conventional casting approach (presence of dark-pigmented spots with uneven pigmentation). The bioprinting strategy facilitates the deposition of cell droplets to emulate the epidermal melanin units (pre-defined patterning of keratinocytes and melanocytes at the desired positions) and manipulation of 3D microenvironment to fabricate a 3D biomimetic hierarchical porous structure. The histological analysis indicates the presence of a well-developed epidermal region and uniform distribution of melanin granules in epidermal region of the 3D bioprinted pigmented skin constructs. Furthermore, the immunochemical analysis reveals that the presence of important biomarkers (col VII, HMB-45, K1 and K6) in the 3D bioprinted pigmented skin constructs, whereas there is only presence of HMB-45 and K6 biomarkers in the manually-cast constructs. Although 3D bioprinting is an advanced manufacturing platform, the incorporation of other important strategies such as macromolecular crowding and co-culture techniques are critical toward the fabrication of 3D pigmented human skin constructs.

6.2. Future recommendations

From this project, it has been shown that 3D bioprinting is a feasible manufacturing method for fabricating 3D pigmented human skin constructs with uniform skin pigmentation. However, more work needs to be done to eventually fabricate fully-functional 3D pigmented human skin constructs. The current tissue maturation duration is a relatively long process (39 days in total) under static culture conditions. The next step could focus on the development of optimal co-culture medium and the use of pro-pigmenting agents/stimuli to accelerate the tissue maturation process. Furthermore, more extensive work can include the use of dynamic culturing conditions to improve the tissue maturation process.

The primary cells in this experiment are purchased from a cell supplier (Promocell), hence it is challenging to quantify the corresponding skin pigmentation on the 3D bioprinted pigmented skin constructs to the respective skin donors. Future collaboration with National Skin Center would enable the quantification of skin pigmentation with corresponding skin donor to analyze the similarity in obtained skin pigmentation. Furthermore, it is important to consider the significant differences between the skin of Asian and Western populations and the way they respond to different chemicals. To test the repeatability of the bioprinting strategy, primary skin cells from skin donors of different ethnic groups could be utilized to develop 3D pigmented skin constructs with different degrees of pigmentation.

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