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Functional tissues from intelligent materials, 3D printing and stem cells

Qi Gu

University of Wollongong

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FUNCTIONAL TISSUES FROM INTELLIGENT MATERIALS, 3D PRINTING AND STEM CELLS

A thesis submitted in fulfilment of the requirements for the award of the degree

DOCTOR OF PHILOSOPHY

from

University of Wollongong

by

QI GU
This work is dedicated to my parents, Mr. GU Ke-Jing and Ms. LI Yong-Ling, my sisters, Ms. GU Jing and Ms. GU Na and my brother, Dr. Gu Zhen for their unconditional love and support.
CERTIFICATION

I, Qi Gu, declare that this thesis, submitted in fulfillment of the requirements for the award of Doctor of Philosophy, in the School of Chemistry, University of Wollongong, is wholly my own work unless otherwise referenced or acknowledged. The document has not been submitted for qualifications at any other academic institution.
ABSTRACT

Regenerative medicine has evolved into the stage of “smart regenerative medicine” where biomaterials can actively influence cell fate and behavior to form and regulate tissue function. Tissue engineering is a prominent tool of regenerative medicine that can utilise stem cells for regenerative therapy because of their capacity to proliferate and differentiate into lineage specific cell types. The status of stem cells is dependent on the extracellular conditions, which include chemical signals such as growth factors, and the properties of extracellular materials in a three-dimensional (3D) environment. With the development of materials science and 3D bioprinting, it is possible to build complicated functional tissues in vitro for regrowth of lost and damaged tissues or organs. Therefore, through “additive manufacturing”, advanced tissue constructs can be fabricated using bioactive and biodegradable materials with integrated tissue-specific cells, whereby the mechanical structure and cell-cell interactions closely emulate in vivo tissue and function.

The work described herein relates to the development of simple and reproducible approaches to constructing neural tissue by bioprinting human neural or induced pluripotent stem cells that are differentiated in situ to functional neurons and neuroglia. The supporting biomaterials (comprising alginate, carboxymethyl-chitosan and agarose) form a novel clinically relevant 3D porous gel by stable crosslinking after printing, which encapsulates stem cells for subsequent expansion and differentiation. Differentiated neurons are spontaneously active, show a bicuculline-induced increased calcium response, and are predominantly gamma-aminobutyric acid expressing. In addition to neural tissue, human induced pluripotent stem cells could be induced to generate the embryoids within printed constructs comprising cells of three germ lineages endoderm, ectoderm, and mesoderm. A second component to this thesis related to the investigation of electrical stimulation via conductive biomaterials for future potential application with 3D bioprinting for tissue engineering. Electric field is one
important parameter involved in the cell growth and embryo development. In support of further controlling/regulating stem cell state, obtained results provisionally indicate that electrical stimulation via a conducting biopolymer augments human iPSCs to differentiate into neuronal cells. While further research will be necessary, the present findings provide support for the development of 3D configured conductive materials to enhance stem cell differentiation to neural and other tissues. Furthermore, conductive constructs may be produced by adapting the presently described 3D bioprinting platform.

In conclusion, the methods described herein provide a foundation to build upon for advanced manufacturing of 3D human neural and other tissues for near-term research of tissue development, function and disease, and longer-term regenerative medicine including transplantation therapy.

**Key words:** Stem cells, Biomaterials, 3D bioprinting, Human tissue engineering, Hydrogel, Tissue modelling, Regenerative medicine.
ACKNOWLEDGEMENTS

Firstly, I want to thank my supervisors Prof. Gordon Wallace and Prof. Jeremy Crook. Prof. Gordon Wallace is a world-renowned scientist in the fields of materials, energy and 3D printing. He has a very wide vision and large picture outlook to propose and direct the project. Prof. Jeremy Crook is a world-renowned stem cell expert, and now his research field has expanded to biomaterials for stem cell regulation and functionalization. Without his day-to-day supervision, there would be lots of time and ways lost. I have also learned a lot from Prof. Wallace’s integral arrangement and perspective highlights, and from Prof Crook’s patience, experience, and high-professionalism.

I thank my first Ph.D. supervisor, a great scientist, Qi Zhou for his continuous support and advice.

Wollongong is a quiet and comfortable city, which keeps the noise from the researchers. The Intelligent Polymer Research Institute is a fantastic place to do research, and there are very advanced and extensive facilities here. The assistance of many individuals (Eva, Kerry, Rodrigo, Toni, Tony, Zhilian, Xiao, Caiyun, Phil…) has been unforgettable. The collaboration and discussions on some interesting scientific topics with many individuals (Yu, Hongrui, Yan, Rodrigo, Long, Javadi, Shazed, Siti, Zhi, Jianfeng, Zan, Yang…) have been invaluable. Thanks to all the people in the institute.

Thank you to the scientists whose works have changed the world and whose thoughts have inspired me.

Finally, thank you to Mum and Dad, whom I admire most in my life, and who have always supported me.

Cantabile Years, Immortal Efforts
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<table>
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<tr>
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<tbody>
<tr>
<td>2D</td>
<td>two dimensional</td>
</tr>
<tr>
<td>3D</td>
<td>three dimensional</td>
</tr>
<tr>
<td>μg</td>
<td>microgram</td>
</tr>
<tr>
<td>μL</td>
<td>microlitre</td>
</tr>
<tr>
<td>ADSC</td>
<td>adipose derived stem cells</td>
</tr>
<tr>
<td>Ag/AgCl</td>
<td>silver</td>
</tr>
<tr>
<td>Ag</td>
<td>Agarose</td>
</tr>
<tr>
<td>Al</td>
<td>Alginate</td>
</tr>
<tr>
<td>BCC</td>
<td>breast cancer cell</td>
</tr>
<tr>
<td>BDNF</td>
<td>brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>CaP</td>
<td>calcium phosphate ceramics</td>
</tr>
<tr>
<td>CMC</td>
<td>carboxymethyl-chitosan</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DBS</td>
<td>dodecylbenzene sulfonate</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagles Medium</td>
</tr>
<tr>
<td>DRG</td>
<td>embryonic stage nine (E9) chick dorsal root ganglia</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylene diamine tetra acetic acid</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>GelMA</td>
<td>gelatin methacrylate</td>
</tr>
<tr>
<td>GPa</td>
<td>gigapascal</td>
</tr>
<tr>
<td>HA</td>
<td>hyaluronic acid</td>
</tr>
<tr>
<td>hASCs</td>
<td>human adult stem cells</td>
</tr>
<tr>
<td>hCMPCs</td>
<td>human cardiomyocyte progenitor cells</td>
</tr>
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<td>Hep3B</td>
<td>human hepatoma cells</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>hiPSCs</td>
<td>human induced pluripotent stem cells</td>
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<td>hMSCs</td>
<td>human mesenchymal stem cells</td>
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<td>hNSCs</td>
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<td>human osteoblast-like cells</td>
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<td>human umbilical vein endothelial cells</td>
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<td>iPSCs</td>
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<td>NCSCs</td>
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<tr>
<td>OPF</td>
<td>oligo (poly(ethylene glycol) fumarate)</td>
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<td>Pa</td>
<td>pascal</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<td>PC12</td>
<td>a cell line derived from a pheochromocytoma of rat adrenal medulla</td>
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<td>PCL-b-PLLA</td>
<td>poly(epsilon-caprolactone)-block-poly(L-lactide)</td>
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<td>PCL</td>
<td>poly(ε-caprolactone)</td>
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<tr>
<td>pDTEc</td>
<td>poly (desaminotyrosyl tyrosine ethyl ester carbonate)</td>
</tr>
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<td>PEG</td>
<td>poly(ethylene glycol)</td>
</tr>
<tr>
<td>PEC</td>
<td>poly(epsilon-caprolactone)</td>
</tr>
<tr>
<td>PGA</td>
<td>polyglycolic acid</td>
</tr>
<tr>
<td>PLA</td>
<td>poly (lactic acid)</td>
</tr>
<tr>
<td>PLCL</td>
<td>poly(lactide-co-epsilon-caprolactone)</td>
</tr>
<tr>
<td>PLGA</td>
<td>poly(D,L-lactic-co-glycolic acid)</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PMEDSAH</td>
<td>poly[2-(methacryloyloxy)ethyl dimethyl-(3-sulfopropyl)ammonium hydroxide]</td>
</tr>
<tr>
<td>PMMA</td>
<td>poly(methyl methacrylate)</td>
</tr>
<tr>
<td>pNiPAAm</td>
<td>poly(N-isopropylacryamide)</td>
</tr>
<tr>
<td>PSC</td>
<td>pluripotent stem cell</td>
</tr>
<tr>
<td>PVA</td>
<td>polyvinyl alcohol</td>
</tr>
<tr>
<td>RBCs</td>
<td>retinoblastoma cells</td>
</tr>
<tr>
<td>SCs</td>
<td>rat Schwann cells, neurolemmocytes</td>
</tr>
<tr>
<td>SFCS</td>
<td>silk fibroin/chitosan</td>
</tr>
<tr>
<td>SRM</td>
<td>smart regenerative medicine</td>
</tr>
<tr>
<td>VSMCs</td>
<td>vascular smooth muscle cells</td>
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Part I

General Introduction and

Characterization Techniques
Chapter 1

Introduction

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1 INTRODUCTION

1.1 Introduction
The rapid growth of organ transplantation demand has led to potential recipients outnumbering donors. Although one million people benefit from organ transplantation globally, the death toll of the patients on the transplant waiting list is 15-30% [1]. Regenerative medicine, a broad field including tissue engineering and cell therapy, is a promising approach to restore the structure and the function of damaged tissues by regenerating cells or tissues [2]. Since the first U.S. Food and Drug Administration (FDA)-approved cell-based therapy product for treating serious burns [3], many significant advances such as inventions of clinical materials have led the field closer to achieving its potential in improving the lives of countless patients. Regenerative medicine or tissue engineering aims to restore original function without or with low transplant rejection by the host by delivering the cells and material constructs fabricated in vitro into the body.

Biomaterials have been used in medical devices such as artificial bones [4], heart valves[5], nerve conduits[6], cochlea [7] and even components of eyes [8]. Currently, an entire heart is undergoing pre-clinical animal model testing [9]. Biomaterials can be divided into three categories: synthetic, naturally-derived and hybrid materials [10]. Over the past 20 years, there has been a transition from the use of synthetic to natural materials in clinical medicine, with the latter possessing more similar mechanical and biological properties to native tissues. Nevertheless, combining cells with biomaterials provides a promising strategy in regenerative medicine because ensuing tissue growth from grafted tissue into the damaged tissues may restore tissue function[11]. Some biomaterials may be used as scaffolds for improved bio-function and/or for cell delivery in cell therapy such as an FDA-approved product OrCel (a bilayer cellular matrix for the cell culture of dermal fibroblast as skin substitutes)[12].
Cells are the basic structural and functional units of organisms, with an ensemble of similar cells forming a tissue and multiple tissues grouping together to form an organ [13]. Therefore, an organ is composed of various cell types, and the function is dependent on the multicellular interaction. 3D printing, also known as additive manufacturing and rapid prototyping, was initially described as stereolithography [14]. It is a methodology that uses 3D computer-aided design (CAD) data to produce a 3D structure, layer by layer. The main features of 3D printing are customisation and high resolution that enables fast and cheap fabrication of a desired structure. 3D printing holds potential to reproduce complex bionic devices or fabricate organs with intricate 3D microarchitecture in vitro. Currently, 3D printing for biological applications has evolved to creating clinical devices for implantation, patterning arrays in drug screening system, cell encapsulation and even reconstruction of an entire organ [15]. Therefore, 3D bioprinting holds great promise for regenerative medicine.

In this section, an overview of regenerative medicine is provided together with the development of biomaterials for research and regenerative medicine and 3D bioprinting in tissue engineering with emphasis on organ and bionic organ construction.

1.2 Regenerative medicine

Regenerative medicine, a term invented in 1999 by William Haseltine, is an emerging interdisciplinary field that involves engineering, replacing, or regenerating human cells, tissues or organs to establish or re-establish normal function to treat diseases and injuries [16, 17]. Stem cells have tremendous promise in regenerative medicine due to their capability of self-renewing and differentiation. Stem cell therapies may include injection of stem cell-derived specialized cells into target sites, infusion of biologically active molecules secreted by stem cells and possible growth of tissues or organs in vitro for transplantations into patients whose damaged organ are no longer able to self-repair [18, 19]. In addition to using
the patient's own tissues or cells to regenerate organs, the regenerated organ could potentially solve the problem of organ shortage and transplant rejection [16, 20].

Regenerative medicine strategies generally fall into two categories: cell-based and non-cell-based regenerative medicine [21]. For non-cell-based regenerative medicine, synthetic materials and biomaterials are utilized to replace diseased tissues or parts in the human body. The advent of new man-made materials such as Teflon and silicone has contributed to a wide array of devices that can be used in humans [22]. Currently, man-made tissue-like devices have already been used in clinical therapies, which include artificial hearts [23] and titanium alloy bones [24]. However, although these devices provided a structural replacement, the function of the repaired tissue was not restored as normal as the native ones[25]. For the cell-based regenerative medicine, cells can be delivered via injection or an appropriate biomaterial scaffold such as hydrogels [26]. Biomaterials provide a 3D environment for cell survival, attachment and new tissue formation with appropriate structure and function[27]. In addition, biomaterials act as media for the delivery of cells and bioactive factors into the desired sites in the body [28]. Following on from this, consideration will now be given in the next section to the application of stem cells and biomaterials.

**1.2.1 Clinical application of stem cells**

Recently, there has been a growing interest in stem cells-based clinical treatment [https://clinicaltrials.gov/] [29], which includes pluripotent stem cells and adult stem cells. Embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) are pluripotent stem cells that can be differentiated into any cell type of the three embryonic germ layers and have self-renewing capability [30, 31]. Adult stem cells such as hematopoietic stem cells (HSCs), marrow mesenchymal stem cells (MSCs) and neural stem cells (NSCs) are derived from specific tissues and retain the multipotent property of differentiating into major specialized tissue cell types.
The ability of stem cells offers a bright future for regenerative medicine. However, it is hard for stem cells to proceed through the safety assessment stage whilst few clinical studies have advanced to phase III. Only a handful have a curative effect on patients, because of the undeterminable differentiation after the graft [32]. Thus, the terminally differentiated cells, which resemble those in the lesion area, may provide a better treatment option with improved safety. In 2010, the first human ESC based therapy product, GRNOPC1, was used for treating spinal injury[33]. Later in 2012, the first human ESCs-derived terminally differentiated cells, retinal pigment epithelium cells, were used to treat age-related macular degeneration, for which two successful results were reported [34, 35]. Nevertheless, many problems in cell treatment remain, for instance, rejected cells can neither interact with the surrounding internal tissues nor reach diseased sites as expected [36]. Several studies have proposed the combination of cells and materials as a solution; especially in treating damaged cells and tissue transplantation [37-40]. Also, materials can act as stem cell regulators, dictating the function of stem cells and can be used to fabricate tissue-like structure at the macro- and micro-level [41]. Consideration will now be given in the next section for the roles of materials in regenerative medicine.

1.2.2 Biomaterials in regenerative medicine

The human body contains various tissues with different shapes and functions. In microbiology, cells are the basic unit of an organism with attachments of extracellular matrix (ECM) to support cell survival and to form functional tissues. Bionic materials are important tools in tissue engineering and are used to replace damaged tissues or mimic the functions of ECM in native tissues. These materials can imitate natural tissues and their environment partially or wholly [42]. Therefore, the research of bionics is a multidisciplinary field combining biology, chemistry, physics, and materials. With hundreds of millions of years of evolution, organisms have the most complex structure over different ranges of scale and
functions. The fact demonstrates not only the importance of the simulation of biological structures but also the functionality when designing bionic tissues or organs.

The history of bionic devices in clinical settings can be divided into three stages (Table 1-1). From the 1960s to 1980s, the first generation of the bionic materials was the fabrication of hard-tissue like parts such as metal bone hammer [43] and ceramic teeth [44] which are still widely used in clinical therapy to date. As synthetic technology advanced, second generation materials were developed in the 1980s which included bio-ceramic [45, 46] and bioactive glasses [47] that could respond to physiological cues. Compared with the first generation bionic materials, there were no toxic side effects, or immune rejection response nor disruption of the immune system, but advantageously were corrosion resistant and possessed high tensile strength [48]. In the late 1990s, a new generation of biomedical materials started to be developed with properties biodegradability and improved influence on cell activities [49]. Currently, new biodegradable biomaterials are used, which could be referred to as intelligent or smart materials as they can respond to the environment [50]. With the involvement of these materials in regenerative medicine, smart regenerative medicine (SRM) has been developed.

Table 1-1 Summary of the three generations of biomaterials

<table>
<thead>
<tr>
<th>The first generation</th>
<th>The second generation</th>
<th>The third generation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type</td>
<td>Ceramics</td>
<td>Bioceramics</td>
</tr>
<tr>
<td></td>
<td>Metal</td>
<td>Bioglass</td>
</tr>
<tr>
<td></td>
<td>Alloy</td>
<td>Biomedical polymer</td>
</tr>
<tr>
<td>Feature</td>
<td>Inert material</td>
<td>Bioactivity or degradable</td>
</tr>
<tr>
<td>Ref</td>
<td>[43, 44]</td>
<td>[45-47]</td>
</tr>
</tbody>
</table>

1.3 Extracellular matrix

The extracellular matrix (ECM) is composed of different types of macromolecules including structural proteins such as collagen and elastin, adhesive glycoproteins such as fibronectin
and laminin and embedded proteoglycans such as hyaluronic acid and other polysaccharides [51]. The general structure of the ECM is shown in Figure 1-1. Cell survival is dependent on the support of the ECM. Nutrients and wastes are transported through the ECM and the conditions outside the cell could be communicated by chemical signals via the ECM, in order to regulate cellular functions. Cell movements including spreading, migration, proliferation, and differentiation are also dependent on the ECM, and thus the ECM plays an important role in cell pattern and cell gradients for tissue development.

Collagen is a chief structural protein in vertebrates, representing 90% of the extracellular protein in bone and tendon. There have been at least 19 types of collagen reported, and they can be classified into fibril-associated collagens (FACIT), network-forming collagens, anchoring fibrils, transmembrane collagens, basement membranes, and others based on the molecular structure. Type I, II, III, V and XI belong to the FACIT group and contribute to the structural backbone and cartilage. Type IV collagens, are the microfibrillar collagens which are involved in the basis of basement membranes and contribute to the dermis, vessel walls, and intervertebral discs. One characteristic feature of all types of collagen is the triple helix; composed of three α-chains. The detailed information of reported collagens is shown in Table 1-2.
The ECM contains structural proteins such as collagen and is embedded with proteoglycan. Fibronectin and laminin are glycoproteins that bind to membrane-spanning receptor proteins called integrins.

**Figure 1-1** Diagram of the ECM
### Table 1-2 Collagen types and distribution

<table>
<thead>
<tr>
<th>Type</th>
<th>Molecular composition</th>
<th>Genes</th>
<th>Tissue distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fibril-forming collagens</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>α1(I)2α2(I)</td>
<td>COL1A1,2</td>
<td>bone, dermis, tendon, ligaments, cornea</td>
</tr>
<tr>
<td>II</td>
<td>α1(II)3</td>
<td>COL2A1</td>
<td>cartilage, vitreous body, nucleus pulposus</td>
</tr>
<tr>
<td>III</td>
<td>α1(III)3</td>
<td>COL3A1</td>
<td>skin, vessel wall, reticular fibres of tissues</td>
</tr>
<tr>
<td>V</td>
<td>α1(V)α2(V)α3(V)</td>
<td>COL5A1-3</td>
<td>lung, cornea, bone, fetal membranes</td>
</tr>
<tr>
<td>XI</td>
<td>α1(XI)α2(XI)α3(XI)</td>
<td>COL11A1-3</td>
<td>cartilage, vitreous humour</td>
</tr>
<tr>
<td><strong>Basement membrane collagens</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>α1(IV)2α2(II)</td>
<td>COL4A1-6</td>
<td>basement membranes</td>
</tr>
<tr>
<td><strong>Microfibrillar collagen</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VI</td>
<td>α1(VI)α2(VI)α3(VI)</td>
<td>COL6A1-3</td>
<td>Descemet’s membrane, skin, heart</td>
</tr>
<tr>
<td><strong>Anchoring fibrils</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VII</td>
<td>α1(VII)3</td>
<td>COL7A1</td>
<td>skin, cervix, cornea, mouth mucosa</td>
</tr>
<tr>
<td><strong>Hexagonal network-forming collagens</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VIII</td>
<td>α1(VIII)2α2(VIII)</td>
<td>COL8A1,2</td>
<td>Descemet’s membrane, endothelial cells</td>
</tr>
<tr>
<td>X</td>
<td>α1(X)3</td>
<td>COL10A1</td>
<td>Hypertrophic and mineralizing cartilage</td>
</tr>
<tr>
<td><strong>FACIT collagens</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IX</td>
<td>α1(IX)α2(IX)α3(IX)</td>
<td>COL9A1,2</td>
<td>cartilage, vitreous humor, cornea</td>
</tr>
<tr>
<td>XII</td>
<td>α1(XII)3</td>
<td>COL12A1</td>
<td>ligament, tendon</td>
</tr>
<tr>
<td><strong>Transmembrane collagens</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>XIII</td>
<td>α1(XIII)3</td>
<td>COL13A1</td>
<td>skin, hair follicle, chondrocyte</td>
</tr>
</tbody>
</table>

Glycoproteins are proteins with covalently attached sugar residues, which are formed of numerous branched oligosaccharide chains and contain 1-60% carbohydrate by weight [52].
Fibronectin is the major cell adhesion glycoprotein with two subunits linked through a pair of disulphide bonds near the C-terminus, which assemble fibrillary networks extended between adjacent cells[53]. As shown in Figure 1-2, each subunit consists of three types of repeating modules: 12 type I (termed FN1, hexagon), 2 type II (termed FN2, square), and 15-17 type III repeats (termed FN3, cylinder) [53, 54]. Fibrillin is a large glycoprotein, which binds elastin and is essential to assemble microfibrils, the cornerstone of the ECM [55]. Laminin is another adhesive glycoprotein, which is a heterotrimeric protein and helps cross-link components of the ECM [56]. Each laminin heterotrimer is comprised of one α, one β and one γ chain with individual genes encoding them [57] (Figure 1-3). The common structural motifs such as a large globular laminin N-terminal domain (LN domain), a laminin coiled-coil (LCC) domain, a rod-like stretch of LE domain and LG domains (LG1–5) at the C-terminus are shared by all the chains [58]. The domains of laminin could bind to perlecan, nidogen, or laminin receptor proteins on the cell membranes. As another of the fibrous proteins/glycoproteins, elastin contains two types of short segments: hydrophobic segments and alanine- and lysine-rich α-helical segments. Elastin chains are formed by the crosslinking of single elastin molecules which could uncoil to an extended conformation when stretched and then recoil spontaneously (Figure 1-4). Elastin is also the precursor of tropoelastin, but the shape and mechanism of the assembly of tropoelastin is still unclear. Some other microfibril proteins such as elastin microfibril interface located protein 1 (EMILIN-1), are derived from elastin and work together with fibulins to give tissue the property of elasticity [52].
Figure 1-2 Diagram of FN subunits.

The figure is reproduced from [53]. Three types of repeating modules are shown for the FN subunits.
Figure 1-3 The structure of laminin.

The figure is from *Sigma* and shows that the classical structure of laminin contains one central \( \alpha \)-chain surrounded by \( \beta \) - and \( \gamma \) -chains.

Figure 1-4 The network of elastin molecules with and without tension.

Covalent bonds (*red*) maintain the cross-linked network. ECM is made up of two main classes of macromolecules: (1) fibrous proteins including collagen, elastin, fibronectin, and laminin, which have been described above and (2) proteoglycans, which are formed of
unbranched polysaccharide chains, glycosaminoglycans (GAGs), covalently linked to the core protein. Proteoglycans can be divided into several families including: the lecticans (aggrecan, versican, neurocan and brevican) which can interact with hyaluronan; the small proteoglycans (decorin, biglycan, fibromodulin, lumican and keratocan) with leucine-rich repeat structures and other proteoglycans which are not well understood such as phosphacan and neuroglycan [59, 60]. The GAGs can be separated into two groups including non-sulphated GAGs, such as hyaluronic acid (HA) and sulphated GAGs, which can be further classified into four groups including hyaluronan, chondroitin sulfate, dermatan sulfate, heparan sulfate and keratan sulfate. GAGs are negatively charged because of their repeated disaccharide sequences, making them the most anionic molecules in animal cells (Figure 1-5). There are three other negative molecules produced in animal cells: phospholipids, nucleic acids, and deoxyribonucleic acid. GAGs are also hydrophilic and can be associated with a huge volume of water after combination with cations. GAGs play a vital role in connection with extracellular molecules to regulate cell signal and development.
Figure 1-5 Repeating disaccharide units of various GAGs.

Four types of non-sulphated GAGs are shown in the figure.

1.4 Biomaterial development for stem cells

Tissue engineering is one area of interdisciplinary research which requires the collaboration of a number of fields including cell biology, materials research and 3D prototyping technologies. Biomaterials are materials with bioactivity when used in cell culture which could facilitate the application of stem cells in tissue engineering [61]. Despite early recognition of the need to preserve or recreate *in vivo* conditions (i.e. the stem cell niche) and characteristics of stem cells *in vitro*, conventional culture methods inevitably alter the cellular microenvironment, inter- and intra-cell signalling, and behaviour, with cells showing different morphologies and the capability to differentiate compared with their *in vivo* counterparts [62-64]. For example, Figure 1-6 shows photomicrographs of the inner cell mass (ICM) of a human embryo at blastocyst stage compared with the ESCs generated from
ICM and cultured in vitro as cell colonies. Unlike the in vivo ICMs, cultured ESCs are maintained under controlled conditions outside their natural environment, unable to interact with other cell types, and endogenous substrates and nutrients, which are substituted with artificial culture platforms and media. Moreover, traditional methods are based on 2-dimensional (2D) culture. Therefore, it is not surprising that the established ESC lines exhibit different patterns of gene expression and cell signals compared to ICM [65]. Newer 3D culture systems, however, are biochemically and physiologically more similar to in vivo conditions.

Figure 1-6 Comparison of ESCs in vivo (A) and in vitro (B).

A shows a human blastocyst, with the dashed circle indicating the ICM, and B shows an ESC colony (black dashed circle) derived from the ICM. Scale Bars as indicated in the figures.

Good stem cell and tissue culture are desirable for the integration of a myriad of components within the in vivo niche to mimic an organism’s biology including mechanical, biochemical, and in the case of excitable cells, electrical properties [66, 67]. Normally, stem cells are cultured and differentiated on polystyrene plates with some wet oxidization or synthetic modification [68, 69]. Advances in tissue engineering using intelligent materials, which could effectively recreate the 3D micro-niche, have provided a solution to bio-fabricating the complex constructs necessary for tissue regeneration and replacement. For example, cancer
cells cultured on 3D scaffolds demonstrated improved cell viability and could be used for drug screening[70]. Fibroblasts, PSCs, MSCs and NSCs have also been cultured on various 3D scaffolds [71-74]. The development of tissue engineering is now booming through the utilization of biomaterials. Advanced functional biomaterials enable the intersection of biology for in situ stem cell actuation, by promoting cell adhesion, survival, proliferation, and differentiation [41]. Importantly, nanomaterials and conductive materials are being used as devices in clinics and in basic biological research, paving the way for the development of advanced tissue engineering in regenerative medicine. Given below is a review of the difference between 2D and 3D culture. The application of 3D materials in tissue engineering and the properties of materials will also be discussed.

1.4.1 2D verses 3D stem cell culture

Most stem cell related research and development (R&D) routinely employs flat-bed 2D culture because of the difficulty of maintaining and characterizing cells using 3D platforms. However, the limitations of 2D culture are obvious when compared with the three dimensionalities of the stem cell niche, whereby in vivo cells are embedded in the ECM excreted by cells, which is organised as a complex 3D structure that ensures cell adhesion and cell-cell-communication and which acts as an optimal store and source of growth factors. To reiterate, the ECM is composed of different types of macromolecules including structural proteins such as collagen and elastin, adhesive glycoproteins such as fibronectin and laminin and embedded proteoglycan such as hyaluronic acid and other polysaccharides (Figure 1-1) [51, 75-77]; although there are many other minor and/or uncharacterized components existing in various tissues. The ECM plays the important role of converting the extracellular conditions into chemical signals to regulate cell growth. Now 3D biomaterials have been developed to imitate the 3D conditions for cell culture.
As Table 1-3 shows, there are two types of 3D cell culture: 3D scaffolds, which are 3D hard structures for cell support and attachment of some parts of the cell surface, and 3D encapsulation which is where the cells are encapsulated into the materials with all parts of the cell surface surrounded by materials. The materials used for 3D scaffolds or 3D encapsulation fabrication have also been indicated at the bottom of the figures. The 3D scaffolds or encapsulation environment could contribute to cell functionalization. In 2D conditions, cell morphology or cell colonies are flat. The same cells grown under 3D conditions were found to grow in multilayer cell structures. The 3D structure significantly influenced the phenotypes of the relative cell types including proliferation and migration [78, 79]. Further molecular characterization reinforced this point. It was demonstrated that 3D conditions could improve the differentiation efficiency of stem cells for osteogenic differentiation and bone differentiation from MSC [80, 81]. For the pluripotent stem cells, 3D conditions have been used to induce iPSC generation [82, 83]. 3D conditions could mimic the physiological environment better than conventional 2D cultures[84].
**Table 1-3** 2D vs 3D for cell culture*

<table>
<thead>
<tr>
<th>Cell type</th>
<th>2D</th>
<th>3D scaffolds</th>
<th>3D encapsulation</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>breast cancer cells (MCF-7)</td>
<td>polystyrene</td>
<td>collagen</td>
<td>peptide hydrogel</td>
<td>[85, 86]</td>
</tr>
<tr>
<td>Neuronal cells</td>
<td>laminin coating glass</td>
<td>PuraMatrix</td>
<td>alginate+carboxy methyl-chitosan+agarose</td>
<td>[87, 88]</td>
</tr>
<tr>
<td>hepatocellular carcinoma cells (HepG2)</td>
<td>polystyrene</td>
<td>PMMA</td>
<td>ExtraCel™ hydrogel</td>
<td>[89-91]</td>
</tr>
<tr>
<td>Human embryonic stem cells</td>
<td>Matrigel ® coating polystyrene</td>
<td>Matrigel ® treated pDTEc</td>
<td>alginate/collagen +Matrigel®+PVA</td>
<td>[72, 90, 92]</td>
</tr>
</tbody>
</table>

Biocompatible and bioactive materials are required for positive interaction with cells [93]. The ECM contains many properties, and so the materials are fabricated with various modifications to meet the requirements for the cellular properties. Numerous polymers and
natural materials extracted from organisms have been used for 3D cell culture (Table 1-3). The nanoscale interface structure could also improve the biocompatibility and bioactivity of the materials with cells [94]. Apart from the topography, the chemical properties are also important for the design of biointerfaces. Peptide sequences have been covalently linked to materials to facilitate cell proliferation and attachment [95, 96]. ECM proteins and growth factors have been patterned onto materials to control cell viability and contact [97]. Some other functional materials with electrical and mechanical properties could also improve the biocompatibility and bioactivity of materials. These properties will be detailed in the Section 1.4.3 below.

1.4.2 Biomaterials for 2D/planar stem cell culture and differentiation

Biological materials were used initially to study native materials including bone, feathers and others [98, 99]. Then these materials were developed to be used for clinical therapy. Subsequently, the materials containing natural-based polymers such as collagen, gelatin, fibrin (fibrinogen), laminin and fibronectin have been modified for cell culture (Table 1-4). Collagen is a common protein in the body. Non-fibrillar collagen such as type IV is found in membranes and connective tissues are rich in fibrillar collagen such as type I and III. Collagen scaffolds composited with type I, II or III have been used for mesenchymal stem cells (MSCs) [100], fibroblasts and cancer cells culture [86] and cartilage and skin engineering [101]. The fibrillary collagen matrices or surfaces have been used for cell-reorganization and in vivo function mimics. Fibroblasts spread to a greater extent on collagen coated plates than those without coating [102]. At the appropriate density, the collagen coated plates induced stem cell differentiation [103]. The expression of cell membrane proteins containing vinculin, integrin, paxillin, and zyxin could interact with collagen matrices. Different cell types and different densities induced the expression different quantities of membrane proteins[104]. Gelatin has been widely used for coating polystyrene flasks and
plates. Feeder cells such as fibroblasts, cancer cells such as Hela cells and endothelial cells adhered to the bottom of plates better and longer when cultured on the gelatin [105, 106]. Fibrinogen, a soluble matrix protein which could be generated from hepatocytes, induced endothelial cells adhesion and spreading in vitro through interaction with specific adhesive receptors [107, 108]. In the presence of fibronectin, the fibroblasts and muscle cells also adhered to fibrin substrates [66]. Laminin has been widely used for the expansion of neural stem cells and neuronal differentiation [109, 110]. Fibronectin promoted the attachment of various cells and the fibronectin coated vessels sustained human ESCs, mouse ESCs and NSCs without differentiation [111, 112]. Matrigel®, derived from mouse tumour cells, supported human ESCs and NSCs survival [113, 114]. Vitronectin is reported to be an alternative to Matrigel® for sustaining human PSCs in the feeder-free system [115, 116]. Human PSCs hold great promise in regenerative medicine however they are difficult to maintain without feeder cells; therefore 2D synthetic surfaces have been developed to expand human ESC culture by using peptide-acrylate [117], PMEDSAH [118], glycosaminoglycan [119] and polyacrylate [120]. Although these surfaces could not sustain the PSCs for as long as feeder cells, this represents a tremendous advance in the combination of materials with human PSCs. The aforesaid 2D polymers possess sought after properties including wettability, roughness, and elasticity. 2D biomaterials have now been used as carriers to locate cells in cell therapy such as the FDA-approved product OrCel for skin replacement [121].
### Table 1-4 2D polymers for cell culture

<table>
<thead>
<tr>
<th>Materials</th>
<th>Cell types</th>
<th>Roles</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nature</td>
<td>Collagen</td>
<td>fibroblasts, MSC</td>
<td>[122-124]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>cells spreading and adhesion</td>
<td></td>
</tr>
<tr>
<td>Gelatin</td>
<td>fibroblasts, endothelial cells, cancer cells</td>
<td>cells adhesion</td>
<td>[106, 125]</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>endothelial cells</td>
<td>cells adhesion</td>
<td>[107, 108]</td>
</tr>
<tr>
<td>Laminin</td>
<td>NSCs, PSCs</td>
<td>cells adhesion</td>
<td>[126]</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>MSC, PSCs, NSC</td>
<td>cells adhesion</td>
<td>[111, 112]</td>
</tr>
<tr>
<td>Vitronectin</td>
<td>PSCs</td>
<td>cells adhesion</td>
<td>[115, 116]</td>
</tr>
<tr>
<td>Matrigel®</td>
<td>PSCs</td>
<td>cells adhesion</td>
<td>[113, 114]</td>
</tr>
<tr>
<td>Artificial</td>
<td>peptide-acrylate</td>
<td>PSCs</td>
<td>[117]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>cells adhesion and cardiomyocytes</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>differentiation</td>
<td></td>
</tr>
<tr>
<td>PMEDSAH</td>
<td>PSCs</td>
<td>cell adhesion</td>
<td>[118]</td>
</tr>
<tr>
<td>glycosaminoglycan</td>
<td>PSCs</td>
<td>cell adhesion</td>
<td>[119]</td>
</tr>
<tr>
<td>polyacrylate</td>
<td>PSCs</td>
<td>cell adhesion</td>
<td>[120]</td>
</tr>
</tbody>
</table>

1.4.3 Biomaterials including alginate, chitosan and agarose for 3D stem cell culture and differentiation

The current scaffolds that have been investigated for cell culture have been listed in Table 1-5 which contains information on the types of scaffolds, the materials employed and the cells studied. It can be concluded that the cells used in the 3D scaffolds are mainly fibroblast-like cells because of their adherent property. The types of scaffolds used depended on the
morphology of the cells. Fibrous scaffolds have been used for the functionalization of stem cells. Neural stem cells can be differentiated into functional neurons [101]. Porous scaffolds are very common scaffolds and have been used for cell proliferation and growth, such as for fibroblasts, chondrocytes and MSCs. There have been reports of human ESCs (H9) cultured on PLGA porous scaffolds free from feeder cells, a system which allowed specific lineage differentiation[102]. Fibrous and porous scaffolds can conveniently bind pre-designed factors and then release them to improve cell manifestation during culture, whereas the normal 2D conditions inhibited the efficacy of this feature [104]. Microspheres can be used to carry culture cells in suspension. They have also been used for embryoid formation from human PSCs and to improve their differentiation efficiency [103]. Custom scaffolds are fabricated using some prototyping techniques such as 3D printing with various morphologies as required. Low bio-compatibility, however, has limited their application. Therefore they are commonly combined with bio-compatible materials, like natural polymers including gelatin, alginate, and collagen [80, 127-130]. Through decellularization, native tissues are converted into ECM scaffolds which have high biocompatibility because of native bio-composition. Scaffolds from hydrogels (made from mannitol, chitosan, collagen, OPF, alginate, HA, PEG, fibrin and others) are very popular in stem cell research. Their biodegradable and biocompatible properties are similar to those of native tissue [131]. In fact, natural polymers used for 2D conventional cultures are also hydrogels, which are suitable for the fabrication of 3D scaffolds [132-135].
Table 1-5 Types of 3D scaffolds used in stem cells study

<table>
<thead>
<tr>
<th>Types</th>
<th>Materials</th>
<th>Cells</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrous</td>
<td>Titanium, PCL-b-PLLA, PCL, Poly (L-lactic acid)</td>
<td>Chondrocytes, PC, DRGs, SCs, ECs, DPSCs</td>
<td>[100, 136-138]</td>
</tr>
<tr>
<td>Porous</td>
<td>PLCL, PLGA, BG, PEC, blend of PLGA and PVA</td>
<td>VSMCs, hOLCs, hMSCs, SMCs, Chondrocytes, H9</td>
<td>[127, 130, 139]</td>
</tr>
<tr>
<td>Microspheres</td>
<td>Gelatin, Collagen, Chitosan, PLGA, PLA</td>
<td>Chondrocytes, NP, MSCs, RB, BBCs, MCF-7, ADSCs</td>
<td>[140-142]</td>
</tr>
<tr>
<td>Custom</td>
<td>PEG, GelMA, PGA, PLA, PCL, glass, hydrogels, CaP</td>
<td>hMSCs, hVESC, BMSCs, C2C12, Osteoblasts</td>
<td>[128, 129, 143-145]</td>
</tr>
<tr>
<td>Native tissue</td>
<td>silk, cartilage, SIS, collagen membranes, Porcine aortic heart valve roots, Trabecular bone, liver</td>
<td>ADSCs, SMCs, hUVECs, hepatocytes</td>
<td>[146-149]</td>
</tr>
</tbody>
</table>

One important class of 3D scaffold enables 3D encapsulation, with the materials used having the potential to be used in tissue engineering because of their bioactive properties. After encapsulation, the cells can be delivered into the site(s) of interest. In addition, different cell types can be used to fabricate tissues by directly curing the hydrogel with different cells at different locations [150]. Table 1-6 includes examples of natural and synthetic materials used for 3D cell encapsulation with natural polymers, such as those polysaccharides and proteins derived from seaweeds and ECMs of animals, most commonly used [151-153]. The components of polymers have specific effects on cell culture, which can also determine the different properties of the materials, which are described in the sections below.
<table>
<thead>
<tr>
<th>Materials</th>
<th>Sources</th>
<th>Crosslinking methods</th>
<th>Cell types</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agarose</td>
<td>polysaccharide from seaweed</td>
<td>Thermally induced bacteria[154], MSCs[155]</td>
<td></td>
</tr>
<tr>
<td>Alginate</td>
<td>polysaccharide from seaweed</td>
<td>Ionic crosslinking ADSC[151], human PSCs[72, 156], Rat heart endothelial cells (RHECs)[157], mouse ESC[158, 159], human MSC[160]</td>
<td></td>
</tr>
<tr>
<td>Chitosan</td>
<td>polysaccharide from crustaceans</td>
<td>Ionotropic crosslinking hybridoma cells[161], hamster kidney cells[152], rat NSCs[162]</td>
<td></td>
</tr>
<tr>
<td>Gelatin or gelatin derivatives</td>
<td>gelatin is denatured, hydrolysed collagen and could be modified by methacrylamide or furfurylamine</td>
<td>Light induced or chemical treatment BMSC[163], HepG2[145], articular cartilage cells[164], human cardiac-derived progenitor cells (hCMPCs)[165],</td>
<td></td>
</tr>
<tr>
<td>Materials</td>
<td>Sources</td>
<td>Crosslinking methods</td>
<td>Cell types</td>
</tr>
<tr>
<td>-------------------</td>
<td>------------------------------------</td>
<td>-------------------------------------------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>Hybrid polysaccharide</td>
<td>mixture of different polysaccharide</td>
<td>Ionotropic/chemical treatment cross-linking</td>
<td>human NSCs[88], L929[168],</td>
</tr>
<tr>
<td>Collagen</td>
<td>natural protein from ECM</td>
<td>Thermal cross-linking/pH variation</td>
<td>MSC[169, 170], NSCs[171]</td>
</tr>
<tr>
<td>Fibrin</td>
<td>natural protein from blood</td>
<td>Enzymatic cross-linking</td>
<td>cardiomyocytes[172], human MSCs[173], ADSCs[167], human umbilical cord stem cells[174]</td>
</tr>
<tr>
<td>HA</td>
<td>Polysaccharide from ECM</td>
<td>Thermal or photo upon chemical modification</td>
<td>human ESCs[175], NIH-3T3[153], chondrocytes[176] ADSCs[177]</td>
</tr>
<tr>
<td>PEG</td>
<td>one artificial polyether</td>
<td>Thermal upon chemical</td>
<td>ADSCs[177], HDFs and</td>
</tr>
<tr>
<td>Materials</td>
<td>Sources</td>
<td>Crosslinking methods</td>
<td>Cell types</td>
</tr>
<tr>
<td>-----------------</td>
<td>--------------------------</td>
<td>------------------------------------</td>
<td>---------------------------------</td>
</tr>
<tr>
<td>ECM</td>
<td>ECM for tissues</td>
<td>Thermal cross-linking or chemical</td>
<td>BMSCs [179], hCMPCs [180],</td>
</tr>
<tr>
<td></td>
<td></td>
<td>treatment</td>
<td>Human iPSC derived neurons[181],</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>hASCs and</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>hTMSCs [182]</td>
</tr>
<tr>
<td>pNiPAAm or</td>
<td>artificial polymer</td>
<td>Thermal cross-linking</td>
<td>human</td>
</tr>
<tr>
<td>PNIPAm based</td>
<td></td>
<td>linking</td>
<td>PSCs [183], ADSCs [184]</td>
</tr>
<tr>
<td>hydrogel</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1.4.3.1 Micro-surface of the scaffold

It is believed that surface topographical features can induce a surface signal through, for instance, integrin-mediated adhesion, to regulate cell movement [185, 186]. Cell attachment is dependent on the roughness of the substrate porosity (Figure 1-7) and the porous substrate is suitable for cell adhesion and extension [187]. Hydrophilic modification of PCL nanofibres can improve the proliferation of neural progenitors and help them to keep their normal morphology [188]. Nanoparticle coated silk nanofibres were more suitable for spreading MSCs than the non-coated silk nanofibres [189]. Recently, nano-topographical surfaces were also used for human ESC colony preservation. This provided a new platform for the study of human ESC feeder-free cell culture [190]. One study systematically explained the effect of
topographic features, ranging in size from 0.4 - 10um, on the neural differentiation from MSCs, and the results showed that the surface with smaller gratings could enhance regulation of neuronal markers such as MAP2 [191]. These reports provide evidence that topographical features can guide cell growth through cytoskeletal arrangement and adhesion of the cells [192]. Cell gradients are important for cell patterning in the human body and biomaterials have also been developed with spatio-temporal gradients to induce tissue regeneration [193]. Precise technologies such as electrospinning [194], laser etching [195], and self-assembly-assisted nanolithography [195] have been developed to produce surface topography with special patterns for cell culture and to promote stem cell differentiation [196]. In addition, the surface charge, chemistry and wettability all play important roles in the determination of cell fate [197].

Figure 1-7 Microscopy of the surface from two cell plates.

A is a Corona plate. B is a CellBind\textsuperscript{®} plate with the latter having greater porosity and larger pores. Both of them are from Corning\textsuperscript{®}.

1.4.3.2 The mechanical properties of scaffolds

The interaction between cells and the elasticity of substrates can influence stem cell fate. Stem cells have low elasticity (about 100–1000 Pa), and human tissues have higher elasticity (about 1000–10,000 Pa) compared to stem cells. The differentiation of stem cells into
specific tissue cells can be improved when cultured on substrates which have elasticities similar to those of the intended tissues [158, 198, 199]. Stem cells can maintain self-renewal on biomaterials with low elasticity such as Matrigel®. The specializations from human MSCs can be directed by the specific stiffness of the matrix, and there have been diverse assays of genetic expression on the substrates with different elastic properties [200]. Other in-depth research has shown that the stiffness of soft hydrogels such as polyacrylamide (PAAm) influences stem cell states by adjusting the pore size in the materials [103]. The specification of stem cells is dependent on the complex ECM environments. The variations of stem cell ECM can affect the activity of membrane proteins. The protein-receptor interaction also contributes to stem cell fate determination. Integrin transmits the attachment into the cytoplasm and the information is transduced into some pathway signals which control cell activities. Many studies have started to explore the mechanisms behind the effects of mechanical properties on cell growth. When the materials are used for substrates of cells, the cells will have one or more contact points with them. As natural materials, collagen, fibronectin, and Matrigel® have been used to change the mechanical properties of substrates for cell culture [201]. Some other synthetic materials as aforesaid also have different mechanical strength, flexibility, and stiffness. In vivo, the mechanical properties of the ECM have important roles to play in determining the structure of tissues and the connection of adjacent tissues by adjusting the network of collagen, fibronectin and fibrin in order to alter stiffness [202]. The mechanical properties are transduced into biochemical signals through the interaction between the ECM and the cells to initiate the cell action [203]. The elastic moduli of tissues range from a few Pa in the brain to a few GPa in the bone [204]. Furthermore, the contractile nature of cells and tissues can be greatly impacted by mechanical properties. Normally, differentiated cells prefer materials with a higher elasticity than those preferred by stem cells. As Figure 1-8 shows, mechanical properties have been used to
promote stem cell differentiation and morphologies [205]. Cell remodeling on substrates with different contraction is determined by myosin-actin and integrin organization [206]. On the stiff substrates, there is a strong development of actin which is involved in the Rho GTPase signaling a pathway to force stem cell differentiation [207, 208].

![Image](image.png)

**Figure 1-8** Stem cells on substrates with different mechanical properties.

A Stem cells can proliferate on the scaffold with lower elasticity

B Stem cells can differentiate when cultured on a hard substrate

1.4.3.3 **Other properties of materials**

Materials could be developed with specific peptide modification to improve cell viability and attachment. The cell adhesive peptide RGD which is derived from integrin-binding and fibronectin has been widely used covalently immobilized with alginate [209], PEG [210, 211] and gellan gum[212] for tissue engineering. The peptides including YIGSR, IKVAV, DGEA, and MNYYNSNS from other structural proteins such as laminin, collagen and vitronectin also hold promise in the bioactivity modification of materials[213]. Additionally, the porous structure in the 3D materials is essential for cell nutrition and migration. Salt leaching, gas foaming, phase separation and freeze-drying are used during fabrication of the scaffolds to control the pore size [214]. Electrical property is also important for materials. Electric fields have been found to have promising potential in promoting cellular events and physiological development. Nervous system repair needs electrical signal communication between the implanted devices and the internal nerve system. Conductive materials such as graphene
could be coated on non-conductive materials to be used as electrodes without influencing the device’s biocompatibility[215]. Conducting materials play important roles in neural prosthesis including cochlear and ocular implants. These materials including polypyrrole, polyaniline and some other conductive polymers are also proven to stimulate neural stem cell differentiation [216-218].

1.5 3D bioprinting

Bioprinting has the potential to reduce the demand for donor organs as it combines different kinds of cells and materials (either naturally-derived polymers or synthetic molecules) to generate various tissues such as heart tissues, blood vessels and cartilages[219]. To print materials with cells simultaneously, hydrogels are often chosen for encapsulating cells. Hydrogels could be printed because the “ink” could be cross-linked after printing by changing the chemical or physical properties. Many reports have considered suitable materials for cell printing including alginate, gelatin and HA. Before crosslinking, the ink has the right consistency and stiffness to meet both the printing and scaffold requirements. PCL, PLGA and some other materials are used to print some scaffolds because they can be melted at high temperature and could be solidified by reducing the temperature [220]. This section mainly summarizes the development of 3D bioprinting and the benefits of its application in regenerative medicine.

1.5.1 Bioprinting technologies

3D printing technology was first described by Charles W. Hull in the 1980s [221] and there have been 30 years of development to date. It is regarded as one of “The top ten fastest-growing production industries in America” [222]. Presently, the use of 3D printing spans various fields, from medicine, textiles, machinery, architecture, military, jewellery to aerospace. 3D bioprinting, on the other hand, is a newly-emerging technology that holds promise in the medicinal field [223]. It is a new interdisciplinary field of regenerative
medicine and tissue engineering, which combines rapid prototyping technology with bio-
manufacturing techniques to fabricate 3D structures, through the layer-by-layer precise
positioning of bioink containing biomaterials and cells. Considering the shortage of donor
organs and the inevitable allograft rejection reaction, 3D bioprinting has many advantages
compared with traditional tissue engineering, such as time-saving, rapid speed, high
resolution, individualization and high levels of mimicry. The main technologies in
bioprinting are inkjet [224, 225], microextrusion [226, 227] and laser-assisted printing [228,
229]. In these systems, inks of cell suspensions are placed in a printer cartridge where a
computer controls the printing patterns. However, the different features such as surface
resolution, cell viability and the biological materials used for printing can influence the
quality of the products of these technologies (Table 1-7). Each printing technology has been
investigated comprehensively and has its own merits and shortcomings as illustrated in Table
1-7 and below [230-237].
### Table 1-7 Schematics and features of selected biofabrication patterning techniques

<table>
<thead>
<tr>
<th></th>
<th><strong>Inkjet Printing</strong></th>
<th><strong>Microextrusion Printing</strong></th>
<th><strong>Laser-assisted bioprinting</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mechanism</strong></td>
<td>Non-contact, using thermal or piezo technology to prompt the liquid droplets</td>
<td>Contact, robotically controlled extrusion of materials</td>
<td>Non-contact, via pulsed laser directly onto gel with cell suspension</td>
</tr>
<tr>
<td><strong>Print speed</strong></td>
<td>1-10,000 droplets/s</td>
<td>10-50 μm/s</td>
<td>200-1,600 mm/s</td>
</tr>
<tr>
<td><strong>Resolution</strong></td>
<td>&gt;50</td>
<td>5-200</td>
<td>1-3</td>
</tr>
<tr>
<td><strong>Cell viability</strong></td>
<td>75%-90%</td>
<td>40%-80%</td>
<td>&gt;95%</td>
</tr>
<tr>
<td><strong>Benefits</strong></td>
<td>High print speed, low cost, high resolution, wide availability</td>
<td>Better resolution spatial controllability and more flexibility in the material</td>
<td>High adaptability with materials, high cell viability, high resolution, clogging avoided, high cell concentrations</td>
</tr>
<tr>
<td><strong>Limitations</strong></td>
<td>Low droplet directionality, nonuniform droplet size, low cell concentrations, materials must be liquid</td>
<td>Slow print speed, printer cost medium, low cell availability, low resolution</td>
<td>High cost, low overall flow-rate</td>
</tr>
<tr>
<td><strong>Ref.</strong></td>
<td>[225, 238, 239]</td>
<td>[227, 240, 241]</td>
<td>[229, 233, 242-244]</td>
</tr>
</tbody>
</table>

#### 1.5.1.1 Inkjet bioprinting

Inkjet-based bioprinting is a non-contact technology. It laminates droplets of biological materials to produce two-dimensional (2D) and 3D structures (Figure 1-9A) [245, 246]. There is difficulty, however, in control of the size and the flow continuity of the ink droplets.
Hydrodynamics need to be considered here. Currently, inkjet bioprinting mainly includes thermal inkjets and piezoelectric inkjets [249], which use thermal or piezo technology to prompt the liquid droplets through the pre-settled 3D structure. The ink in the cartridge is replaced with biological materials including cells, cell culture fluid or gel precursors. The development of inkjet printing technology has resulted in the achievement of a relatively suitable quality at a low cost that affords broad application prospects in 3D cell printing. The technology can be used in high-throughput bioprinting but it is difficult to achieve control over single cells [250].

1.5.1.2 Microextrusion bioprinting

Microextrusion bioprinting is the most common 3D printing system, in addition to being more affordable. Unlike inkjet printers, which generate droplets, the microextrusion printer’s materials are deposited onto a substrate (Figure 1-9B). Directed by CAD software, small beads of materials are deposited into two dimensions where the deposited layer serves as a foundation for the subsequent layer while the stage or microextrusion head moves along the z-axis hence forming a 3D structure. The most common way to extrude biological materials for 3D bioprinting is through the use of pneumatic [251-253] or mechanical (piston or screw) systems [254]. The cell survival rate decreases with increasing extrusion pressure and nozzle gauge [255]. This observed decrease in cell viability is mainly due to the shear stresses inflicted on cells in viscous fluids. Although cell viability can be increased by using low pressures and large nozzle sizes, this also results in decreased resolution and printing speed.

1.5.1.3 Laser-assisted 3D bioprinting

Laser-assisted bioprinting uses the laser optical tweezers effect of trace substances sink and thermal shock to deposit droplets containing cells (Figure 1-9 C) [243]. After several decades of development, as a non-contact, sterile technology with high precision and high accuracy, the significance of laser treatment in cell therapy has finally been recognised. According to
the principles adopted by cell deposition, laser printing can be divided into two distinct technologies: laser-induced direct writing (laser-guided direct writing, LGDW) and laser-induced metastasis (Laser induced forward transfer, LIFT)

LGDW was proposed by Renn et al. in 1999 [237]. The laser beam may be in a parallel or perpendicular orientation to generate a force in a direction so that the cells will move in horizontal or vertical directions. When the force of the laser beam on the cells is greater than 10pN, the cells may move within the range of several tens of micrometres to several millimetres, which then will be deposited on the surface of the selected object.

LIFT utilizes laser-ablated materials as the basis of the ink. When the laser beam is directed through the transparent substrate onto the interface of the thin film (transferred material) and matrix, causing an interaction between the laser and the material to be transferred (cell suspension film and material liquid), a trace amount of thin material is forced away from the base body and deposited on the bottom of the substrate-receiving layer[256].

![Figure 1-9 Three types of 3D bioprinting technology for depositing cells](image)

**A** Direct inkjet 3D bioprinting. **B** Microextrusion 3D bioprinting. **C** Laser-mediated 3D bioprinting.
1.5.2 The process of 3D bioprinting

To construct organs using 3D bioprinting, the first step is imaging and modeling. The most common medical imaging technologies are computed tomography (CT) and magnetic resonance imaging (MRI), for which CAD combines the mathematical format to assist with the modeling [15, 246, 257].

For printing preparation, there is a need to use a bioink that is compatible with various types of cells and a matrix which can support proliferation and functionalization of cells. The material selection is based on the material’s bioactivity, physical properties, biocompatibility, toxic degradation and printability. Presently, scientists can successfully extract cells from donors’ bone marrow, adipose or some other tissues and then these cells can be expanded into sufficient numbers for the preparation of printing. Through layer-by-layer deposition from vertical or horizontal, it is possible to complete the organ’s construction [258]. Finally, the materials should be chosen in accordance with the organ’s properties.

After modeling and material selection, there is a need to encourage tissue fusion, remodeling and acceleration of tissue maturation. As organs are composed of different types of tissues, there is a requirement to develop a vascular tree for the organs including capillaries and microvessels. In vitro, bioreactors can be used to maintain tissues and provide maturation-promoting factors. Once the organ is mature, it is ready for transplantation. The process is outlined in Figure 1-10 [259, 260].
Figure 1-10 The process for 3D bioprinting.

Step 1: Imaging and model designing.
Step 2: Selection of cells (up) and materials (bottom).
Step 3: Printing 3D structure and application

1.5.3 Biomaterials for 3D bioprinting

Initially, 3D printing technology was designed for rapid prototyping using earlier materials such as metals, ceramics and thermo-plastic polymers that generally used organic solvents, high temperatures or non-biocompatible crosslinking [15]. Characterization of the physical properties (i.e. porosity, elastic modulus, degradation, and water-swelling) and cell response parameters (i.e. cell viability, proliferation, differentiation and spreading) is fundamental to determining the suitability of these polymers for different tissue engineering applications [261]; hence the variety of biomaterials used in tissue engineering. This section summarizes
the biomaterials used for solid scaffolds and hydrogels, the two main forms used for 3D printing.

Solid scaffolds are the earliest scaffolds used in tissue engineering, especially in bone substitution [4]. To date, solid scaffolds have been studied and used for the regeneration of various tissues in *vitro* or *vivo*. Since the invention of stereolithography, scaffolds can be printed from inks and cured to support cell culture after printing [14]. However, the potential risks and regulatory requirements should be fully understood before the use of solid scaffolds in clinical applications. Although raw materials such as PLA, PEG and PCL have been approved by the FDA, the processing unit for the industrial devices must be optimized and tested for FDA approval [261].

Hydrogels are hydrophilic polymeric network groups that have been used as artificial ECM to encapsulate cells. Some biomaterials could be formed into hydrogels as shown in Table 1-6. Due to their high absorption properties, hydrogels have received significant attention for cell biology and tissue engineering applications [262]. Furthermore, hydrogels possess excellent biocompatibility and possible bioactive molecule motifs encoded in their chemical structures. Hydrogels are widely used for different biomedical applications, such as regeneration, drug delivery, and tissue adhesives [263]. Their features can allow cells to travel in the space of hydrogels and provide room for cell growth and migration. The easier diffusion of nutrients and wastes could avoid cell starvation and cell damage in the absence of vascularization [264].

### 1.5.4 3D bioprinting for regenerative medicine

The goal of regenerative medicine is to develop new ways of restoring the function of damaged and abnormal tissues. It is difficult for traditional methods to construct tissues comprising the many typical features of biological tissues, such as the complex organization of various cell types, complex ECMs and microvasculatures. Nevertheless, 3D bioprinting
can print constructs containing a variety of ECMs, and these biological materials could be organized into uniform or non-uniform layers at different loci of the printed structure to mimic the native tissues [219]. In recent years, advances in the 3D fabrication of biological structures include the fabrication of artificial bladders [265], skin [121], tracheas [266] and heart valves [5] for clinical applications. These examples relied on thin, biodegradable scaffolds which were filled with autologous cells from patients, hence there were no allograft rejections in the patients, and thus alleviated the need for organ transplantation to an extent. In the near future, 3D bioprinting will potentially play a wider role in regenerative medicine because its advantages could contribute to thick tissue construction with complicated structure, which has been described above.

1.6 Stem cells and biomaterials for tissue engineering

Stem cells attract great attention for tissue engineering because of their ability to rejuvenate damaged tissues and to be specialized into functional cell types [267]. At the initial stage, PSCs have been studied for differentiation into embryoid bodies (EBs) which contain the cell types representing three germ layers. Furthermore, the PSCs could develop into neurospheres comprised of neural precursors through suspension cultures of differentiated EBs [268]. Through modification of the culture system, there was self-organized cortical tissue formation from human ESCs [269]. It is exciting to note that a three-dimensional organoid culture system has been developed for cerebral tissue generation from human PSCs, aiming to model the human brain [270]. Now, complete lenses could be produced using endogenous stem cells in a petri dish in vitro for patients [271]. The aforementioned tissues (Figure 1-11A-D) have promoted the application of stem cells. However, the tissues are simple or contain just a few cell layers. It is difficult to generate tissues with blood vessels using conventional methods. Therefore, biomaterials and advanced technologies such as 3D printing are helping tissue engineering. Figure 1-11E, F show an entire liver produced from
the decellularized liver scaffold and the thick tissue generation with lumens by 3D printing [272, 273].

**Figure 1-11** The development of tissue regeneration from stem cells.

A) EB and neurosphere formation from human iPSCs, Bars, 100 µm. B) Cortical tissues from human ESCs on day 46 post-differentiation with cortical plate marker Bf1, TuJ and Ctip2 expression[269]. Bars, 100 µm. C) Cerebral tissues from human PSCs with Reelin expression indicating the presence of Cajal–Retzius cells which are important for cortical plate generation[270]. Bars, 200 and 100 for the left and right panel respectively. D) Left, phase-contrast of a lentoid body from lens epithelial stem/progenitor cells (LECs) on the day 30 post-differentiation. Right, a lentoid body demonstrating magnifying properties[271]. Bars, 100 µm. E) Left, matrix from decellularized whole liver. Right, liver from recellularization of the matrix with about 50 million hepatocytes [273]. Bars, 200 µm. F) Vascularized tissue
Biomaterials are increasingly being used in tissue engineering. Polymeric scaffolds have been widely used in tissue regeneration/restoration through permanent or temporary replacement of the damaged tissues[274]. Scaffolds including PGA, PLLA, PU and PLA have been widely used clinically [275]. However, the tissue types supported are limited falling short of complex tissues with high bioactivity. Therefore, hydrogel-based scaffolds have received considerable interest in tissue engineering because of their high bioactivity and the possibility of creating vascularized tissues[276]. Here, Table 1-8 summarizes the application of these materials in tissue engineering.

**Table 1-8 Biomaterials used for tissue engineering**

<table>
<thead>
<tr>
<th>Materials</th>
<th>Types</th>
<th>Application description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polymer</td>
<td>synthetic polymer</td>
<td>PGA as scaffolds for bone internal fixation devices [277], PLLA as fibres for blood vessel conduits [278], PLGA as meshes for skin graft [279]</td>
</tr>
<tr>
<td>Alginate</td>
<td>natural polymer</td>
<td>scaffolds with amniotic stem cells for bone [280], alginate encapsulating chondrocytes for the ear [281], encapsulating porcine aortic valve interstitial cells (PAVIC) and work with PEG-DA scaffolds for the aortic valve [282]</td>
</tr>
<tr>
<td>Chitosan</td>
<td>natural polymer</td>
<td>scaffolds as skin substitute [283]</td>
</tr>
<tr>
<td>Gelatin</td>
<td>natural protein</td>
<td>encapsulating BMSCs for osteochondral tissue [163], magnesium calcium phosphate gelatin scaffolds for bone[284], gelatin sponge for trachea[285],</td>
</tr>
<tr>
<td>Collagen</td>
<td>natural protein</td>
<td>Alginate/collagen scaffolds encapsulated with</td>
</tr>
<tr>
<td><strong>Materials</strong></td>
<td><strong>Types</strong></td>
<td><strong>Application description</strong></td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
<td>-----------------------------</td>
</tr>
<tr>
<td>Fibrin</td>
<td>natural protein</td>
<td>scaffolds for cardiac tissue engineering [289], encapsulating cardiomyocytes for cardiac tissue [172], work with bioglass as scaffolds for the repair of osteoporotic defects [290],</td>
</tr>
<tr>
<td>HA</td>
<td>natural polysaccharide</td>
<td>scaffolds for skin [291], carriers as corneal endothelial cell (CEC) sheets for cornea [292], scaffolds for central neural tissue [293]</td>
</tr>
<tr>
<td>Multilaterals</td>
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<td>gelMA encapsulating MSCs with PCL, PVA and alginate as scaffolds for anatomically shaped constructs [240], fibrin-collagen encapsulating chondrocytes with PCL as scaffolds for cartilage [294], methacrylated hyaluronic acid (Me-HA) and methacrylated gelatin (Me-Gel) hydrogel encapsulating human aortic valvular interstitial cells (HAVIC) for heart valve [295], alginate, chitosan, fibrin and gelatin encapsulating ADSCs for liver [167], gelatin and HA patches encapsulating hCMPCs for preservation from myocardial infarction [165]</td>
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**Figure 1-12** also presents examples of biomaterials application in tissue engineering to describe the development of the field. Biomaterials have been developed from scaffolds for cell culture for the fabrication of one entire organ, with the combination of stem cells and the assistance of rapid prototyping (RP) technologies. With the involvement of stem cells, wanted tissue would be generated *in vivo* [280]. 3D printing technology has been improved as
a powerful tool for tissue engineering [296]. It could be used to distribute cells precisely and to print tissue with high resolution [281]. Successful 3D printed tissues have been used in vivo from hCMPCs and HA/gelatin based matrices [165].

**Figure 1-12** Biomaterials for tissue engineering.

A) Human iPSCs in 3D collagen scaffolds [82]. B) The bones formed from implanted constructs with amniotic fluid–derived stem (AFS) cells, diamond, scaffolds seeded with AFS cells, triangle, scaffolds without AFS [280]. C) Left, tissue-engineered trachea from gelatin sponge. Right, the scaffold implanted into the dorsal subcutaneous spaces of nude mice [285]. D) Human skin equivalents reconstructed from human TERT-immortalized keratinocytes and fibroblasts (right panel) with the native skin (left panel) and primary human skin equivalent (middle panel) as control. Hematoxylin and eosin (H&E) staining was used for histological appearance and vimentin staining was used for fibroblasts [297]. E) Fabrication of HA-CEC sheet constructs. Left, cell sheet (asterisk) attached to HA scaffold.
Middle, Hoechst 33258 staining showed the cross-section of the construct with large and fine arrows pointing large and small pores on the carrier respectively, asterisk, cell sheet, bars, 200 μm. Right, cornea implanted with HA carriers (up) and HA+cell sheets (bottom), bars, 5 mm [292]. **F**) Visible transplanted hCMPC scaffold as a patch on the infarcted area of the ventricular wall on 1 (left) and 4 (middle) weeks with Troponin I (TnI) staining for CMPC and human-specific Lamin A/C expression[165]. **G**) 3D printed bionic ear (left, bars, 1 cm) with coil antenna (right up, bars, 5 mm) contacting the neocartilaginous tissue with viability (right bottom, bars, 50 μm) [281].

1.7 3D printing used for tissue repair

An ideal 3D bioprinting system is shown schematically in **Figure 1-10**. Firstly, accurate information on tissues and organs should be collected for designing the model. Secondly, the server should be able to convert the information into electrical signals to control the printer to print the tissues, and the printer should be able to maintain the cell viability during the fabrication process. Usually, tissue is composed of many types of cells and the cells will be mixed with some substances to be better fused [298-300]. At present, some hard tissues can be fabricated with bionic materials by a 3D printer [301], and these have been used in clinical trials. A 3D printer cannot construct a complicated tissue at present. In the future, a 3D bio-printer could be used to print organs for repairing damaged body parts or to simulate some functional tissues for research, therapy and drug screening. Furthermore, 3D bioprinting can be used for personalized therapy with reduced costs. Biocompatible and biodegradable materials can be combined with 3D bioprinting to reduce the incompatibilities caused by materials [299, 302]. The sections below will detail the application of 3D bioprinting in tissue engineering.
1.7.1 Hard tissue repair

Hard tissue is mainly composed of solids such as collagen and substituted hydroxyapatite with ceramic [303]. Therefore, acellular tissues such as bone and teeth could be repaired using biomaterials more easily than the cellular tissues could be repaired.

Infections, external force, abnormal bone development and other bone defects caused by diseases have serious impacts on patients’ daily lives. Advances in bone tissue engineering for treating bone defects provide new ways to take advantage of the rapid prototyping technology [304, 305]. Injuries occur uniquely and high resolution is required for the interlinkage. Therefore, it is difficult to replace and repair damaged tissues using traditionally fabricated scaffolds. To overcome this issue 3D printing, a highly personalized technology, has been initiated to create a precise bone mold for individual patients [305]. A cancer patient, Erig Moger, most of whose face was removed surgically initially relied on a feeding tube in order to eat [306]. Later, his doctor used CT and facial scanning technology to scan the patient’s skull, followed by a construction of a normal 3D face model using 3D printing and nylon plastic. The screws for the artificial face were also created with a 3D printer. After combining this with autologous bone, 3D printing was used to treat orbital floor fractures as one cost-effective technique [307]. The successful surgery, incorporating of the new artificial hard tissues enhanced the quality of life of the patient greatly by enabling the patient to eat, drink and see normally and renewed his life prospects. Similar to human bones, the structure and morphology of teeth are complex with diverse organizational structures. Dental growth and development in adapting to the alveolar structure are also complex. Therefore, traditional tissue engineering technology for tooth regeneration has encountered several complexities [308]. At present, growth factors and autologous cells have been considered for use in hard tissue printing to enhance tissue bioactivity [309]. One particular study showed that one thermosensitive microparticulate material could be combined with cells to print strong
constructs for bone repair [310]. These results will facilitate future cell-based hard tissue printing.

As 3D bioprinting technology is a computer-assisted modeling technology designed to meet an individual’s needs, the technology also has great potential in stomatology surgery.

1.7.2 Soft tissue repair

Soft tissue connects, supports or surrounds all structures or organs in the body. Traumatic injury or tumour resections often require a large amount of soft tissue reconstruction. From the aesthetic or cosmetic point of view, soft tissue reconstruction is also important for the patients to maintain a good quality of life [311]. This section will review the soft tissues in skin, nervous and vascular systems, which are the three main research foci in soft tissue regenerative medicine. Many studies have been conducted to reconstruct these tissues [15].

Skin is the largest organ of the human body. Skin losses due to wounds or burns require a transplant to protect the wound, which can be difficult due to the lack of autologous or allosome skin [312]. Over the past four decades, industry and academia have invested in and designed the engineering of human skin [313-315]. Initial efforts focussed on the development of a skin graft for wounds and obtained significant results. Following on from that, the research focus progressed to the development of a skin model in vitro and the permeability of drugs and excipients [316, 317]. However, the typical approach to engineering skin begins by simplifying its complexity, and this cannot render it as the normal layered structure. Tissue engineering has a high potential for the production of new skin. Several skin substitutes like Integra® and Matriderm® have already been used in clinical settings as supplements of autologous split-thickness skin grafts [318-320]. Nevertheless, the main difficulty still lies in the reconstruction of the subcutaneous microvascular network and sweat glands. However, 3D bioprinting may be able to solve the problem and pattern cells as for the native tissue[321]. The structure of human skin has been printed using fibroblasts and
keratinocytes [322, 323], however the development and functional factors involved need further investigation [324]. Neural tissue is the main component of the central and peripheral nervous systems, known for its difficulty in spontaneous recovery [325]. Biological substitutes such as conductive polymers and biomaterials for the maintenance, restoration or improvement of neural tissue function have been indispensable to neural regenerative medicine [326, 327]. Moreover, the interaction between cells and biomaterials plays an important role in tissue fabrication because it improves cell proliferation and functionalization, which has been described in the previous sections. Initially, efforts were put into the binding of the small functional molecules to scaffolds for the promotion of nerve regeneration and the results in animal models were encouraging [328]. Furthermore, cells co-cultured with biomaterials, a novel technology combination, have been used in peripheral nerve and brain injury repair [329]. A novel polysaccharide-based hydrogel for neural stem cell printing has recently been developed which included functional mini-neural tissue construct formation [88]. To mimic the ceramal cortex structure, hand-held printing methods were used to print primary neuronal cells in addition to which there was neuronal network observation [212].

The blood vascular system is the main mode of transport in tissue. The main challenge in tissue engineering is limited mass transfer [330]. Some simplex and thin tissues (i.e. skin [38], cartilage [294]) have been preliminarily established. However, due to the size of tissue engineering, the vascular system needs to be incorporated into the tissue structure for the supply of nutrients and oxygen to cells. To this end, a 3D inkjet bioprinting system was used with NIH 3T3 mouse fibroblasts in tubes, which resulted in an overhang structure with post-printing cell viability of over 82% [331]. A microvascular device was developed comprising cylindrical microchannels with medium flow and endothelial cells attached on the side walls[332]. Then, a large network of blood vessels located in a microvascular perfusion unit
was used to support the self-assembly and connection to an existing network [333].
Previously, fibroblasts and umbilical vein endothelial cells combined with material scaffolds
were used for blood vessel regeneration [334]. Currently, there are also novel computer aided
algorithms and methods developed for 3D bioprinting of scaffold-free biomimetic
macrovascular structures (self-supported model) [40]. To supply nutrients and transport the
waste from the thick tissues and organs, sacrificial materials such as pluronic F-127 were
used in initial 3D printing, which were later removed by dissolving them with fluids. The
constructs retained the tube shapes needed for medium perfusion [272, 335].
Many challenges in 3D bioprinting concerning tissues and organs need to be addressed.
However, the preparation time for the bioink before printing is dependent on the time it takes
to obtain a sufficient amount of cells which is usually lengthy when using conventional cell
culture methods, and is also dependent on the cell type [40]. In addition, materials such as
synthetic or bio-based polymers are required for an extremely precise development to match
with the natural tissue. The tear strength, bursting strength, mechanical and biological
properties, blood compatibility and long-term stability are the primary limiting factors in
creating large structures; as there is no single material that meets these requirements.
Therefore, researchers are turning their attention to combining several materials to obtain a
well-rounded biomaterial for 3D printing and tissue culture, or to the exploration of new
functional materials [336].

1.7.3 Complicated tissue fabrication in vitro
3D printed prosthetics [337], jaw bones and tracheas [338, 339] have been used in clinical
settings with good functionality. Moreover, 3D cell spheroids or hepatocytes are being used
to build a liver model and support artificial devices [340]. Unfortunately, there is still no solid
organ that can be used for clinical transplantation although organ models have been
constructed for surgical stimulation in vitro with structures exactly mirroring true organs. To
print organs with essential vascular networks for nutrient and waste transportation, several studies have successfully constructed a vascular tree into the printed organ [341]. Nonetheless, many challenges must be met, as described above, before a truly functional artificial tissue or tissue analogs can be generated.
1.8 Reference


60


[207] E. Yim, M.P. Sheetz, Force-dependent cell signaling in stem cell differentiation, Stem cell research & therapy 3(41).


Chapter 2

General Experimental
2.2 Reagents and Materials

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2.3 Synthesis of hydrogels and conductive polymers

2.3.1 Al-CMC-Ag preparation

Alginate (Al), also called alginic acid or algin, is a type of polysaccharide, a linear copolymer with homopolymeric blocks composed of (1-4)-linked uronate residues (Figure 2-1). Al obtained from brown algae is afforded, in different types, with different viscosities; one of which with a viscosity of 100–300 cP was chosen [1]. Chitin is a homopolymer (poly[β-(1→4)-2-acetamido-2-deoxy-D-glucopyranose]) produced from the shells of marine animals. The idealized structure is shown in Figure 2-2. Chitosan is a copolymer (poly [β-(1→4)-2-amino-2-deoxy-D-glucopyranose-2-acetamide-2-deoxy-D-glucopyranose]) which is derived from chitin by deacetylation with alkali. The process is schematically shown in Figure 2-3 [2]. One serious problem with chitosan is its poor solubility in neutral solutions. Carboxymethyl
chitosan (CMC) is obtained from the carboxymethylation of chitosan, which can be divided into O- and N-carboxymethylation, with the latter chosen for this work because it is a well-defined derivative[3]. Agarose (Ag) is also extracted from seaweed and is a purified linear galactan. Due to the structure of agarose containing D-galactose and 3,6-anhydro-L-galactopyranose, agarose is thermosensitive (Figure 2-4)[4].

Figure 2-1 Molecular structure of alginate

Figure 2-2 Molecular structure of chitin

Figure 2-3 Molecular structure of chitosan
Ag was dissolved in sterile PBS by micro-waving for several seconds. The vials were then placed on a stirrer, 5\% (w/v) Al was added and stirred at 60 °C for 30 min, followed by the addition of 5\% (w/v) CMC and another stirring step at 60 °C for 1 h. The final solutions were allowed to cool to RT. The solutions can be stored in the refrigerator for up to 3 weeks.

2.3.2 Synthesis of the PPy-DBS conductive polymer

The pyrrole monomer and DBS were purchased from Sigma. The pyrrole monomer was distilled before use and stored at -20°C, aliquots of which were taken immediately before they were required. The monomer formula is C₄H₅N with a molecular weight of 67.09g/mol. The structures of the monomer and polymer are shown in Figure 2-5. The synthesis of PPy-DBS was achieved through the oxidative polymerization of pyrrole at an anode. Aqueous monomer solutions were prepared with distilled and deionized water (ddH₂O, Milli-Q) with 0.05 M DBS. Gold-coated mylar (Solutia Performance Films), where the oxidative polymerization occurred was sterilised with isopropanol followed by drying under a N₂ stream. The aqueous solution was degassed using N₂ before PPy-DBS films were grown on mylar films. The traditional two electrode electrochemical cell was used for the oxidation galvanostatically at 0.1 mA/cm² for 10 mins on the platform eDAQ EA161 potentiostat (eDAQ Pty Ltd, Australia) (Figure 2-6). The area for deposition needed to be determined prior to the synthesis for calculation of the constant current to be applied; using knowledge of the height of the solution and the width of the working electrode.
2.4 Stem cell culture and differentiation

Human NSCs are the precursors of various types of neuronal and glial cells. Human iPSCs are one type of pluripotent stem cell with the potential to generate any type of cell of the body. Both were used to test the bioactivity of hydrogels and the synthetics that were generated in the work.
2.4.1 Human NSC culture and differentiation

The human NSC cell line used was purchased from Millipore (SCC007). The human NSC culture medium was prepared by mixing NeuroCult® NS-A Basal Medium (Human) supplemented with heparin (2 μg/mL), epidermal growth factor (EGF, 20 ng/mL), and basic fibroblast growth factor (bFGF, 20 ng/mL). The human NSC cryogenic vials were removed from the liquid nitrogen storage container, allowed to thaw in the water bath at 37°C. Human NSCs were seeded at a density of 2-3 x 10⁶ cells per well of a low attachment six-well plate (Corning) with two mL human NSC culture medium per well. During culture, the human NSCs formed into neurospheres spontaneously. Following five to seven days of culture, the medium containing spontaneously formed neurospheres was collected for digestion with TrypLE (pre-warmed to 37°C), which could then be used for bioprinting. If cells were to be further subcultured, the cells were seeded on low-attachment plates. The normal neural spheres are shown in Figure 2-7.

![Figure 2-7 Neurosphere formation during culture of human NSCs.](image)

The photomicrograph shows human NSCs following 3 days of subculture.

Human NSCs were differentiated in a human NSC differentiation medium containing two parts DMEM/F-12 and one part neurobasal medium supplemented with 2% StemPro, 0.5%
N2, and brain-derived neurotrophic factor (BDNF; 50 ng/mL). NSCs were incubated on the laminin (20 μg/mL) coated plates for differentiation and the medium was refreshed with the differentiation medium every three days. The differentiated human NSCs are shown in Figure 2-8.

Figure 2-8 SEM image of differentiated human NSCs.

The differentiated human NSCs exhibit long neurite extensions.

2.4.2 Human iPSC culture and differentiation

Working stocks of human iPSCs were maintained as 2D cultures in 5% CO2 at 37°C, mTeSR™1 on Matrigel® basement membrane matrix in 6-well plates (Greiner Bio-One). Cells were passaged when colony centres became dense by incubation with 0.02% EDTA for 3 min and fluxing with a pipette, followed by a 1:4-1:6 split. To form EBs, iPSC colonies were extracted non-enzymatically and transferred to fresh culture plates for non-adherent suspension culture in the medium without bFGF.

For 3D printed human iPSC culture and differentiation, mTeSR™1 was again used for human iPSC expansion, and the KOSR medium (DMEM/F12 supplemented with 20% KnockOut™ Serum Replacement, 1×MEM non-essential amino acids solution (NEAA; 100x stock) and 55 μM β-mercaptoethanol) without bFGF was used for induction of EBs. The
initial culture medium was supplemented with 5 µM rock inhibitor Y27632 for the first 3
days of culture, while for EB formation, the medium was changed to bFGF-free medium on
the 5th day post-printing. Figure 2-9 shows the conventional colonies of human iPSCs.

![Image of a colony of human iPSCs](image)

**Figure 2-9** A colony of human iPSCs.

The colony is flatted with a distinct boarder.

**2.5 3D printing stem cells and stimulation of human iPSCs**

Before the current study, there had not been any report on 3D bioprinting for human NSCs
and human iPSCs. This section will describe human NSC and human iPSC culture and
differentiation under two different conditions including 2D and 3D environments, as well as
under electrical stimulation. The platform used for 3D bioprinting was the 3D-Bioplotter by
EnvisionTEC (Figure 2-10).
2.5.1 3D printing human NSCs

Prior to printing, the ink and cells were prepared. The human NSCs were collected as described in the section on stem cell culture (2.4). 5x10^6 cells were suspended in 0.5 mL bioink. The samples were loaded into a 55cc syringe barrel (Nordson) and centrifuged at 300 g for 1 min to remove air bubbles. The design of the 3D model (eg. 10 mm x 10 mm x 2 mm) was accomplished using Blender™ software and saved as a STL type file. The STL file could be converted to a bp format file using Bioplotter® RP software. The bp file was then opened to establish a new protocol for the 3D printing. The applied pressure for bioink printing was set at 1.5-2.0 bar, and the temperature of the barrel and platform was set at 15 °C on the Bioplotter® RP software according to the manual. Stainless steel dispensing tips (Nordon, 7018333) were used for dispersion. After printing, the printed scaffolds were immersed in 2 %
w/v calcium chloride for 10 min for crosslinking. After crosslinking, the scaffolds (Figure 2-11) were immediately washed using culture medium three times for 1 min each, followed by two 10 min washes. Then the scaffolds were incubated for one h in the medium prior to conducting extended tissue culture.

Each construct was incubated in one well of a 6-well plate with NSC differentiation medium at 37 °C for a minimum of 10 days post-printing, with half-volume medium changes performed every 2-3 days.

![Printed Al-CMC-Ag with human NSCs.](image)

2.5.2 3D printing human iPSCs

The process of 3D printing human iPSCs is similar to that of NSC printing. 2x10⁷ cells were collected and then suspended in 0.5 mL gel to comprise the bioink. After mixing the biomaterials with the cells, the bioink was introduced into 55cc Syringe Barrels (Nordson) with stainless steel dispensing tips (Nordon, 7018333) for 3D printing. After printing and gelation, the constructs needed to be rinsed using culture medium three times, followed by two 10 min washes and equilibration in culture medium for 1hour to remove excess calcium ions. To differentiate iPSCs into neurons in the 3D printed structure, the medium was changed to neural induction medium (comprising DMEM/F12 supplemented with 1×N2 supplement, 2 µg/mL heparin and 1×MEM non-essential amino acids solution (100x stock))
on the 5th day after printing. Two weeks later, the medium was changed to a neuronal
differentiation medium containing 2 parts DMEM/F-12: 1 part Neurobasal medium
supplemented with 2% StemPro, 0.5% N2 and 50 ng/mL brain-derived neurotrophic factor
(BDNF) for culture up to 3 weeks. Additionally, all the media for 3D printed constructs
contained penicillin (100U/mL) and streptomycin (100 µg/mL) to prevent contamination. The
culture medium was supplemented with five µM rock inhibitor Y27632 for the first three
days of culture to improve the recovery of human iPSCs [5].

2.6 Physical characterization

2.6.1 Mechanical measurement

The indentation, compression modulus and consistency are crucial to ink printability. They
were measured on an EZ-S mechanical tester (Shimadzu, Japan). Modulus was determined
using both compression and indentation tests as previously described[6]. The hydrogel
solutions were cast in custom-made molds (cylindrical, 10 mm ID, 4 mm in thickness). After
crosslinking, the samples were placed on an EZ-S mechanical tester, then the 10 N load cell
was used to compress the sample with a strain rate of 2 mm/min (Figure 2-12). The
compression modulus (Ecomp) could be calculated from the strain-stress curve using
Equation 2-1. Here, the r equaled 5 mm.

\[
E_{\text{comp}} = \frac{F}{\pi r^2}
\]

2-1
Figure 2-12 Schematic diagram of the mechanical test using compression mode.

During compression of the hydrogel the electrical value increased up to the point of the gel splitting.

Indentation testing was performed using a flat stainless steel indenter (1 mm in diameter) with a 2 N load cell to indent the samples at a rate of 0.1 mm/min. The indentation modulus ($E_{\text{ind}}$) can be calculated from the recorded force ($F$), indentation depth ($d$), indenter radius ($a$) and the reduced modulus ($E_r$) (Figure 2-13). The derivation process is as below [7]:

$$F = 2aEr \frac{d}{a}$$

2-2

Because $E_r$ can also be expressed by the indenter modulus ($E_1$) and the substrate modulus ($E_2$) [8],

$$E_r^{-1} = (1 - \nu_1^2)E_1^{-1} + (1 - \nu_2^2)E_2^{-1}$$

2-3

where $\nu_1$ and $\nu_2$ are the Poisson’s ratios of the indenter and the substrate and the value of $(1-\nu_2^2)E_2^{-1}$ was insignificant when the steel indenter was much stiffer compared to the hydrogel substrate. Finally, Eqn (2-2) and Eqn (2-3) became:

$$F \sim \frac{8}{3}aE_2d$$

2-4
Bioink consistency was measured using previously described method [6, 9]. The scheme of the consistency test is shown in Figure 2-14. The gel was prepared without crosslinking and then loaded into the syringe and the plunger was connected to the upper clamp of the tester. The data was collected during extrusion of the ink.
2.6.2 Scanning electron microscopy (SEM)

In this study, there were two instruments used in the SEM tests: a JSM-7500FA LV Scanning Electron Microscope (JEOL Ltd, USA) and a JSM-6490 LV Scanning Electron Microscope (JEOL Ltd, USA). Prior to SEM imaging, the gels were fixed with 3.7% paraformaldehyde (PFA) for 30 min. Then gels were freeze-fractured by immersion in liquid nitrogen for 60 seconds followed by fracturing using a cold razor blade. Fractured gels were examined with magnifications of 200, 500 and 1000× on a JSM-6490 LV Scanning Electron Microscope. To analyse the surface morphology, the gels were freeze-dried overnight. After depositing 20 nm gold coating to avoid charge accumulation, the morphology of the samples was examined using a field emission scanning electron microscope, JSM-7500FA LV Scanning Electron Microscope.

2.6.3 Weight loss experiment

This experiment was conducted to determine the change in weight over time. The samples were prepared as mentioned above and then vacuum freeze-dried overnight. The weight of the dried sample was determined as \( W_0 \). The dried samples were soaked in a culture medium and incubated in a humid incubator at 37°C and 5% CO\(_2\) for two days. The weights of the dried samples in the determination process were repeated and determined (\( W_e \)). The weight loss (\%) of hydrogels after the two day culture can be described according to the equation[10] below:

\[
\text{Weight loss (\%)} = \left(\frac{W_0 - W_e}{W_0}\right) \times 100
\]

2.6.4 Hydrogel swelling ratio

Swelling is one important property of the hydrogel which can be used for absorption[11]. For the swelling studies, the hydrogels were prepared in the same manner as would be used for cell printing. The initial hydrogel without crosslinking was weighed (\( W_i \)) and then
crosslinked. Monitoring of the time during crosslinking was initiated and there were three time points chosen for the tests: 15 min, 60 min, and 180 min, at which times the gels were weighed (\(W_e\)). The following equation then determined the swelling ratio:

\[
Swelling\ ratio = \frac{W_e - W_i}{W_i}
\]

2.6.5 Diffusion experiment

Diffusion of solute into and out of the gels was characterized by using a similar method to that applied by Lozano R [12]. Three cylindrical hydrogels of 2 cm x 0.35 cm diameter were soaked in solutions containing fluorescently labelled bovine serum albumin (FITC-BSA, Sigma), then the uptake and release of the protein was calculated by collecting the solution and measuring the loss of solute over time (0, 0.5 h, 1 h, 2 h, 4 h, 6 h, 8 h, 10 h, 12 h, and 14 h) until the equilibrium was reached. We determined the FITC-BSA concentration using a micro-plate reader (Fluostar Omega, BMG Labtech). Concentrations of BSA were calculated from a standard curve. The hydrogels were kept at a constant temperature of 37 ºC in a shaking water bath. The release was calculated after the gel reached equilibrium by soaking the gels in fresh PBS, and taking fluorescence measurements over time. Diffusion coefficients of FITC-BSA in the gels were calculated using a nonlinear regression method, reported by Li [13], Carman and Haul[14] and Crank [15] according to the Equation 2-7:

\[
\frac{C_t}{C_\infty} = 1 - \sum_{n=1}^{\infty} \frac{4\alpha(1 + \alpha)}{4 + 4\alpha + \alpha^2 q_n^2} \exp\left(-\frac{D t q_n^2}{r^2}\right)
\]

2-7

where:

- \(C_t\) is concentration of BSA in the bath at time \(t\) (g/cm³)
- \(C_\infty\) is concentration of BSA in the bath at equilibrium (g/cm³)
- \(\alpha\) is \(K\times\) (bath solution volume)/(gel solution volume)
$K$ is the equilibrium partition coefficient, $= C_\infty^{gel}/C_\infty^{bath}$

$D$ is the diffusion coefficient of BSA in the gels (cm$^2$/s)

$r$ is the radius of the gel cylinder (cm)

$q_n$ is the positive, nonzero roots of the equation $a q_n J_n(q_n) + 2 J_1(q_n) = 0$, where the $J_n$ are Bessel functions of $n$th order.

2.7 Electrochemical characterization

2.7.1 Cyclic voltammetry

Cyclic voltammetry (CV) is a potentiodynamic electrochemical technique to probe the nature of electrochemical redox processes. An electrochemical cell with three electrodes consisting of a reference electrode, working electrode and counter electrode, was setup as shown in Figure 2-15. The basic principle is that the redox reaction at the working electrode induces the current between the working and counter electrodes. The potential at the working electrode is applied by a potentiostat between an initial value and a final value (generally -0.6V to +0.8V vs. reference electrode) at a fixed scan rate. The current at the working electrode was monitored and a plot of current versus the applied potential was recorded and displayed by the software which was supplied by EDAQ.
2.7.2 Electrical stimulation

Electrical stimulation was performed on custom-made Plexiglas chambers which were attached to the PPy-DBS films as the working electrode with non-toxic silicone adhesive (Flowable silicone sealer, ITW Permatx Inc, USA) and the counter electrode was the custom plastic cover with auxiliary platinum mesh (Figure 2-16). The device was immersed in distilled water overnight and then sterilized by immersion into 70% ethanol for 5 min followed by drying in the hood. The whole system used for stimulation is shown in Figure 2-17. Digital Stimulator, DS8000 generated the biphasic waveform, and an A365 Isolator (World Precision Instruments) was used for the current control interfaced with the eDAQ system to record the signal.
**Figure 2-16** Custom two electrode setup for stem cell stimulation.

The module comprised a PPy coated gold working electrode and a platinum mesh counter electrode.

**Figure 2-17** The stimulation system components

Cell chamber connected to an isolator and parameters for stimulating set using the stimulator.
2.8 Cell analyses

2.8.1 Human NSC and human iPSC viability assay

Viability assay could be used to display cell dispersion in the gels. Calcein AM is one cell permeant dye which reacts with intracellular esterases in the live cells to be converted into green-fluorescent calcein. Propidium iodide (PI) is a membrane impermeant dye which binds to the double stranded DNA. The samples were treated with calcein AM at 37 ºC for 10 min followed by PI for 1 min. The 3D constructs were mounted onto glass slides and a coverslip placed over the slide-mounted construct using glue. Finally, the slides were reversed and then observed on a confocal microscope (Leica TSC SP5 II). ImageJ can calculate the number of the live and dead cells.

2.8.2 Cell proliferation analysis in the 3D printed gels

PrestoBlue® is one resazurin-based cell viability test reagent, which is reduced to resorufin by NADPH, FADH, FMNH, NADH and cytochromes in viable cells and the reduction process will induce fluorescent forms [16]. In the current study, the samples were first incubated with the reagent, then the supernatant was collected into a 96-well plate and screened by a microplate reader (POLARstar Omega) to obtain the fluorescent intensity. The gels without cells were used as the blank control.

2.8.3 Cell Immunophenotyping

Cell immunophenotyping is one immunohistochemistry reaction for determination of protein expression. Samples were fixed using a 3.7 % paraformaldehyde (PFA; Fluka) solution in phosphate-buffered saline (PBS; pH 7.4). The samples were blocked and permeabilized overnight at 4 ºC with 5% (v/v) donkey serum in PBS containing 0.3% (v/v) Triton X-100. Constructs were incubated with conjugated antibodies (in the dark) or unconjugated primary antibodies at 4 ºC overnight. Constructs incubated with unconjugated primary antibodies were immersed in 0.1% Triton X-100 in PBS three times followed by incubation for one hour.
at 37 ºC with an Alexa Fluor-conjugated secondary antibody. Staining was performed with DAPI at RT for 10 min followed by treatment with an antifade reagent. Then the constructs were mounted onto glass coverslips and imaged for qualitative analysis with a confocal microscope.

2.8.4 Live cell calcium imaging

Intracellular calcium signals generated by calcium ions regulate many cell functions such as muscle cell contraction and neurotransmitter-containing synaptic vesicles [17]. Calcium imaging is one important method for analyzing neuronal function/activity. In this study, Fluo-4 was used to detect calcium flux. Constructs were incubated with Fluo-4 (2 µM) in a fresh culture medium for 30 min at 37 ºC, rinsed in Tyrode’s solution, and mounted on coverslips for the observation of spontaneous intracellular calcium release on a confocal microscope; ideally while immersed in Tyrode’s solution. Bicuculline, a GABA(A) receptor antagonist, was added to induce intracellular calcium release and the image was immediately acquired through a confocal microscope. Leica AF software can be used to quantify the signal intensity.

2.8.5 Reverse transcription-quantitative PCR (RT-qPCR)

RT-qPCR is used to detect and quantify the gene expression at the RNA level. It contains two steps: one is to reverse the mRNA into cDNA; the other is to quantify the cDNA. For RT-qPCR, a trizol reagent was used to isolate the total RNA of the samples, with the purity of RNA defined by NanoDrop™. RNA is transcribed to cDNA with random primers in the RNAse-free system and qPCR was performed with a Gotaq 2-step RT-qPCR system (Promega) on the Bio-Rad CFX real-time instrument.
2.9 Reference


Chapter 3

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3 Bioink development for stem cell printing

3.1 Introduction

The strategy of encapsulating cells in biodegradable materials offers many advantages including ease of handling, mimicking the *in vivo* tissue-like environments and processability of delivering the materials into sites of interest [1]. High water-content hydrogels are widely used in this strategy [2]. A wide range of hydrogels have been used to encapsulate cells for 3D bioprinting as described in Section 1.1. At present, alginate, chitosan, gelatin and hyaluronic acid (HA) etc. have been used for cell encapsulation culture [3]. To permit immediate seeding and uniform cell distribution, 3D printing technology is used in combination with hydrogels for cell patterning, which is known as bioprinting [4-6]. Another advantage of bioprinting is that this technology can pattern the “ink” in precise geometries. During processing of cell-hydrogel mixtures, the hydrogel must support cell viability while maintaining printable properties. Gelatin-methacrylamide (gelMA, a protein-based hydrogel modified from gelatin) has been used for 3D bioprinting of several cancer cell lines [7]. To improve the viscosity, HA and various other components can be added[8]. Importantly, a simple polymer cannot satisfy all the requirements for cell compatibility and printing. Also, a cell’s survival is dependent on attachment through adhesion sites. Although there has been considerable research relating to cell attachment under 2D conditions, there are few reports relating to 3D printable materials.

Initial studies within this PhD relate to the use of a novel 3D polysaccharide-based bioink comprising alginate (Al), carboxymethyl-chitosan (CMC) and agarose (Ag) for printing and encapsulation of human stem cells. Al is a natural polysaccharide derived from brown algae and linear copolymers containing (1,4)-linked β-D-mannuronate (M) acid and α-L-guluronic acid (G) residues[9]. Only the G blocks of adjacent polymer chains are believed to be cross-linked with multivalent cations (e.g., Ca$^{2+}$ or Ba$^{2+}$) through intermolecular actions with
carboxylic groups to form hydrogels[10]. So the combination of the polymers and variations in the solidifying time will determine the mechanical properties of the gel and then influence the cell growth. The Al property is suitable for the 3D bioprinting process. Chitosan is derived from the deacetylation of chitin and has been used in pharmaceutical and non-pharmaceutical fields, but its limited solubility has been a hurdle to its applications[11, 12]. Carboxymethylation is one method which can improve its water solubility [13]. The carboxymethyl group was the biological group chosen because ester and hydrocarbon have been reported to play roles in stem cell growth [14, 15]. Agarose is also a polysaccharide derived from seaweed and is soluble in water when the temperature is above 65°C. Agarose gels are stable and do not swell until heated to 65°C [16]. So during the construction of scaffolds, it can supply support and be used widely for nucleic acid separation [17]. Previous reports have described the concentration of alginate used for 3D printing [18], therefore this chapter is focussed on the optimization of the Al concentration and CMC and Ag for bioprinting.

3.2 Experimental

3.2.1 Bioink synthesis

The general process of developing bioink from Al-CMC-Ag was described in Section 2.3.1. Here, Al-CMC-Ag was prepared with different concentrations of Ag. Different concentrations of Ag solution (including 0.5, 1.5 or 2.5% w/v) in vials were prepared in phosphate-buffered saline (PBS; pH7.4) by heating in a microwave oven, with agitation every 5 sec. This agitation was carried out 11-14 times for the 2.5% and 3-5 times and 5-8 times for the 0.5% and 1.5% respectively. When the agarose was completely dissolved, 5% (w/v) alginate was added into the agarose medium and stirred at 60°C for half an hour. Finally, CMC with different concentrations (w/v) were prepared in the above Al-Ag medium with a
magnetic stirrer at 60°C for an hour. Then the solutions were ready to be used for printing and mechanical testing.

3.2.2 Consistency and mechanical measurement

The experimental procedures have been described in detail in Sections 2.6.1. Briefly, compression and indentation tests can indicate the modulus of Al, Al-Ag and Al-CMC-Ag. Solutions were cast in custom-made moulds (cylindrical, 10 mm ID, 4 mm in thickness). After preparation of the samples, the Al-CMC-Ag solutions were loaded into a 1 mL syringe and measured on an EZ-S mechanical tester (Shimadzu, Japan) with a 10 N load cell at a 0.2 mm s\(^{-1}\) constant strain.

For compression, there was no culture and each sample was mounted on an EZ-S mechanical tester. Then the 10 N load cell was used to compress the sample with a strain rate of 2 mm/min. At least three different samples represented one group of the materials. The compression modulus \(E_{\text{comp}}\) could be calculated from the strain-stress curve, \(E_{\text{comp}} = F/(\pi*r^2)\). Here, \(r\) equalled 5 mm.

Indentation tests were also performed on the EZ-S mechanical tester but a flat stainless-steel indenter (1 mm in diameter) with a 2 N load cell was used to indent the samples at a rate of 0.1 mm/min. The indentation modulus \(E_{\text{ind}}\) can be calculated from the recorded force \(F\), indentation depth \(d\), indenter radius \(a\) and the reduced modulus \(E_r\).

3.2.3 Integrity test

The samples with different concentrations of the CMC were prepared in custom-made moulds (cylindrical, 10 mm ID, 4 mm in thickness). After a washing process, the samples were cultured in the medium without cells. Then, on the second day, the morphology of the hydrogel was observed on a Leica stereo-microscope.
3.2.4 Weight loss experiment

The general method for this experiment has been described in Section 2.6.3. Here, the samples of Al-CMC-Ag with different CMC concentrations (0, 1.5%, 2.5%, 3.5% and 5%) were prepared as detailed in Section 3.2.1. The weight of the dried samples was determined as $W_0$. The dried samples were soaked in a culture medium and incubated in a humid incubator at 37°C and 5% CO$_2$ for 2 days. The weight of the dried samples determination was repeated and the mean value determined as $W_e$. The weight loss (%) of hydrogel can be calculated according to Equation 2-5.

3.2.5 SEM of 3D samples

General processes were described in Section 2.6.2. For surface porosity studies, samples were submerged in human NSC culture media for 24 hr, freeze-dried overnight using a Christ Alpha 2-4 LD Freeze Dryer, then coated with 20 nm gold using an Edwards sputter coater, and kept desiccated until analysed. SEM was performed using a JSM-7500FA LV Scanning Electron Microscope. For studies of internal porosity with and without cells, samples were fixed with 3.7% paraformaldehyde (PFA, Fluka) for 30 min, immersed in liquid nitrogen for 60 seconds, and then freeze-fractured using a cold razor blade. The fractured samples were immediately observed on the JSM-6490 LV Scanning Electron Microscope.

3.2.6 3D encapsulation culture of human NSCs

The Al-CMC-Ag materials with different CMC concentration were prepared as described in Section 3.2.1. $5 \times 10^6$ cells were suspended in 0.5 mL bioink and then crosslinked with 2% calcium for ten minutes followed by rinsing to reduce the high concentration of calcium as follows: 3 rinses with culture medium every minute after crosslinking, a fourth and fifth rinse with culture medium after 10 and 20 mins and a final rinse with culture media 60 mins after crosslinking. 2D culture of human NSCs has been described in Section 2.4.1. The same media employed for 2D and 3D human NSC culture.
3.2.7 Statistical Analyses

Statistical analyses were performed in OriginPro 2015 (Version b9.2.272) using one-way analysis of variance (ANOVA) with Bonferroni multiple comparison post hoc test or two-way ANOVA with Bonferroni post hoc test. Homogeneity of variance tests was performed to confirm that statistical assumptions were met for ANOVA. Statistical significance was set at $P < 0.05$.

3.3 Results and discussion

3.3.1 Synthesis of Al-CMC-Ag

In this study, Al, CMC and Ag were used for synthesizing bioink (Figure 3-1). Al is a polysaccharide derived from algae which can be crosslinked through exposure to divalent cations. Many studies have demonstrated the utilization of alginate for the entrapment of mammalian cells [10]. Recently, there have been reports that the mechanical stability and elastic modulus of alginate can be modified to support neural lineage survival and differentiation [19]. CMC is a derivation of chitosan. Without CMC, after culture, there were cracks on the surface of the hydrogel of Al and Al-Ag (Figure 3-2). The cracks were generated because air bubbles could not escape and there were also some factors from swelling. Ag concentrations were varied but the printing test with the lower and higher viscosities resulted in poorly defined scaffold structures at the same pressure, so 1.5 percent was chosen (Figure 3-3).

Figure 3-1 The chemical structure of Al, CMC and Ag
**Figure 3-2** The integrity of Al, Al-Ag and Al-CMC-Ag.

The left panel represents the hydrogel immediately after crosslinking and the right panel shows the hydrogel after 1 day within culture media. Cracks were observed (white arrows indicate cracks).

**Figure 3-3** Agarose could improve the printability of materials.

Three concentrations of Ag (0.5%, 1.5% and 2.5%) were used for the 3D printing test.
3.3.2 CMC is used to improve human NSC viability

Different concentrations of CMC were tested. The indentation experiment was used to test a localised part of the sample surface. Noticeably, a decrease in modulus was observed with the increase of CMC. But, there was no apparent difference between Al and Al-Ag samples. After 1-4 days culture in cell culture medium, all samples showed a sharp drop in modulus. Al-CMC (5%)-Ag demonstrated an obvious decrease in modulus compared with the other two Al-CMC-Ag samples from 4 days culture (Figure 3-4).

Initial studies of human NSC survival and proliferation within the printed gel construct showed CMC content of the gel influenced human NSC viability, with 5% w/v associated with greater cell loading (indicated by area measurements of individual cells and cell aggregates) compared with lower concentrations ($F (3, 580) = 22.77, P < 0.0001$). Furthermore, there was a significant difference in the effect of CMC content with increasing days of culture ($F (11, 580) = 13.82, P < 0.0001$). Following 9 days culture of 5% w/v CMC-gel constructs, Bonferroni post hoc analysis revealed that cell support was significantly greater than gel constructs with lower CMC content at any time during culture ($P < 0.0001$; Figure 3-5, Figure 3-6). However, support by gel constructs with 5% and 3.5% w/v CMC content at day 5 culture was greater than Al-Ag alone (ie. no CMC) at day 9 of culture ($P < 0.01$ and $P < 0.05$ respectively; Figure 3-5, Figure 3-6). Whereas cell loading of 5% w/v CMC gel increased for the duration of culture (indicative of human NSC proliferation), loading decreased from the time of printing for gels with no or 2% w/v CMC, while 3.5% w/v CMC gel supported an initial increase up to day 5 with subsequent reduced cell loading apparent by day 9 post-printing (Figure 3-5, Figure 3-6). Area measurements of viable cells and aggregates in the gels comprised a wide range of values (manifested as large standard deviations, Levene’s Test (Absolute deviations), $F (11, 580) = 7.59, P < 0.0001$), indicative of a large range of human NSC aggregate sizes (Figure 3-6). Importantly, the population
variances were not significantly different according to Levene’s Test (Squared deviations; $P = 0.68$ ($F(11, 580) = 0.75$)), satisfying homogeneity of variance testing for statistical analysis by ANOVA.

Figure 3-4 Indentation modulus of Al, Al-Ag and Al-CMC-Ag.

Al-CMC-Ag contained different concentrations of CMC. All the materials were maintained in cell culture medium for over 13 days. Data represents mean ± S.D.
Figure 3-5 Survival of printed human NSCs with different percent of CMC.

Live (Calcein AM) and dead (Propidium iodide; PI) human NSC staining at specific time points following direct-printing in gels with different concentrations of CMC. Human NSCs are visible as single cells and aggregates of cells.
The constructs depicted in Figure 3-5 including single cells and aggregates of cells were measured by the area of Calcein AM staining within constructs. Mean ± S.D.; n = 3. Two-way ANOVA with Bonferroni multiple comparison post hoc test. *P < 0.05 (3.5% w/v CMC day 5, vs Al-Ag day 9); **P < 0.01 (5% w/v CMC day 5, vs Al-Ag day 9); ***P < 0.0001 (5% w/v CMC day 9 vs all comparisons).

3.3.3 Further characterization of Al-CMC-Ag

After confirmation of the cell viability in Al-CMC-Ag, it is essential to define why the 5% CMC group had higher cell viability compared with the others. There was a small difference, where the modulus decreased when comparing Al-CMC-Ag with Al and Al-Ag (Figure 3-7). This could be due to the addition of the CMC. Furthermore, the weight loss experiment demonstrated that, 5% CMC led to the increased weight loss of hydrogel from 1.5% to 9% (Figure 3-8). To further define a suitable crosslinking for the new hydrogel Al-CMC-Ag, the compression modulus of the materials was tested for different periods of crosslinking on day 0 and day 4 post cultures, which supported the choice of 10 min crosslinking (Figure 3-9).
The weight swelling ratio showed that the weight of Al-CMC-Ag attained equilibrium after 3h of crosslinking when there would be 40% weight swelling (Figure 3-10), which differed from the swelling ratio of Alginate which could reach more than 200% [20].

**Figure 3-7** Compression measurements of Al, Al-Ag and Al-CMC-Ag.

The samples were tested by compression and data represents mean ± S.D. with at least three repeats.

**Figure 3-8** Weight loss of different materials after one day degradation in PBS.

a, b mean significant difference at P<0.05.
Figure 3-9 The time course of indentation modulus of Al-CMC (5%)-Ag.

Samples with different crosslinking time were tested at day 0 and day 4 after culture in the medium. Mean ± S.D.; n = 3.

Figure 3-10 Swelling properties of Al-CMC-Ag.

The wet weight increase of Al-CMC-Ag at several time points during incubation in PBS buffer.
3.3.4 The mechanism of CMC (5%) improvement of cell viability

To correspond with the weight loss experiments, gel porosity was examined in the absence of cells by low vacuum scanning electron microscopy (SEM), with freeze fracturing for internal analysis. Surface scanning of gels comprising different concentrations of CMC indicated variable porosity, with 5% and 3.5% w/v CMC associated with a highly and sparsely porous surface respectively, and 2% or less w/v CMC gels associated with negligible to no pores (Figure 3-11A). SEM of the gel interior revealed an assembly of polyhedral pores throughout regardless of CMC content, although a range of pore diameters was observed (Figure 3-11B). The alginate gel (Al) displayed a homogeneous porosity which progressed to closed (isolated) porosity as agarose was added to the alginate (Al-Ag); the internal structure (porosity) was changed by increasing pores sizes, as observed. On initial addition of CMC, Al-CMC(0.5%)-Ag and Al-CMC(2%)-Ag, no effect on the porous structure was observed. However, as higher concentrations of CMC were added, such as Al-CMC(3.5%)-Ag and Al-CMC(5%)-Ag, an immediate effect on the internal structures was observed; it seemed to have resulted in a wider pore size distribution together with the appearance of more connected, tunnel-like structures, which has been related to better gas, liquid and nutrients accessibility to cells [21]. Therefore, gels with 5% and 3.5 % w/v CMC comprised a network of larger and smaller sized pores, with the smaller pores often connecting the larger pores. In contrast, gels with 2% or less w/v CMC principally comprised larger pores, with relatively few small pores. A possible model was presented for the Al-CMC-Ag (Figure 3-12). During culture, CMC could be deposited into the medium and the nutrients and oxygen could enter the structure while the whole scaffold was completely retained.
Figure 3-11 Characterization of porosity of gels.

A) SEM showing surface porosity of gels with different concentrations of CMC. B) SEM showing internal porosity of gels with different concentrations of CMC.

Figure 3-12 A model diagram showing the Al-CMC(5%)-Ag structure during culture.

The figure indicates some of the CMC dispersed into the culture medium and some remained in the structure during culture.

3.4 Conclusion

In conclusion, optimizations of the concentration of agarose and carboxymethyl chitosan were carried out to produce printable and bioactive bioink. The mechanical properties were evaluated and the best combination was 5% alginate with 5% carboxymethylchitosan and 1.5% agarose. The use of CMC improved cell viability and distribution. The proposed mechanism
was that some part of the CMC dispersed into the medium during culture, which could then contribute to freedom of transport of the nutrients and waste. Further work in this thesis will detail the diffusion property of the material and its application in the 3D printing of human NSCs and human iPSCs.
3.5 References


Chapter 4

Functional 3D Neural Mini-Tissues
from Printed Gel-Based Bioink and
human NSCs

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4 Functional 3D neural mini-tissues from printed gel-based bioink and human NSCs

4.1 Introduction

3D bioprinting to generate functional tissues has been made possible by recent advances in printing technology, materials science and stem cell science. Also known as additive biofabrication, 3D bioprinting has provided a paradigm shift in in vitro tissue engineering, as a potential remedy to limited supply of functional tissues for modelling development and disease, and transplantation therapy[1]. Bioprinting enables specification of extracellular features and cell organisation for increased control of 3D tissue fabrication. Compared with traditional 2D methods of cell culture in monolayers, 3D printed cultures better recapitulate the natural cell environment and cell-cell interaction for more authentic, reliable and clinically relevant tissue generation. Key features of a printed construct include porosity for diffusion of oxygen and nutrients, and correct mechanochemistry of component biomaterials to promote cell adhesion, survival, networking and function [2].

Strategies for additive tissue fabrication include printing of biomaterial scaffolds that are seeded with cells following printing [3], or concomitant (co-) printing of biomaterials and cells resulting in encapsulated cell constructs[3-9]. The strategy of co-printing offers many advantages including immediate integration of cells with printed biomaterials, more rapid production of a construct, and more authentic simulation of the in vivo tissue environment whereby cells are completely surrounded by and in direct contact with extracellular components and other cells. These features serve to provide a simpler, more automated and defined approach to biomaterial-cell interfacing for reproducible, robust and germane construct development.

Here we report a well-defined and reproducible method for making a novel 3D neural mini-tissue construct (nMTC) by microextrusion bioprinting frontal cortical human NSCs with a
supporting bioink followed by in situ differentiation to functional neurons and supporting neuroglia. The bioink comprises polysaccharides Al, CMC and Ag, which form a gel by chemical crosslinking following extrusion with human NSC encapsulation. Al and Ag provide structural support for the construct, with Al enabling gelation in the presence of cations after printing and Ag conferring suitable bioink viscosity during printing prior to gelation. CMC is a water-soluble derivative of chitosan and conducive to cell survival within the construct. Human NSCs can be maintained as self-renewing cells following printing, continuing to proliferate in situ for approximately 10 days. Differentiation of human NSCs principally results in GABAergic neurons, together with glial cells expressing astrocyte and oligodendrocyte lineage markers. Importantly, neurons are spontaneously active and show a bicuculline-induced increased calcium response, indicative of the presence of receptors for GABA and therefore GABA responsive neurons, and consistent with the occurrence of aforesaid GABAergic neurons. Finally, the method will enable interrogation of neural development, function and disease and may be adaptable for generating other neuronal and non-neuronal MTCs in vitro. Moreover, the MTCs have the potential to be used to develop larger macro-tissue constructs by either “rational design”, “autonomous assembly” or both[1].

4.2 Experimental

4.2.1 Human NSC culture and differentiation

2D culture methods have been described in Section 2.4.1. For 3D human NSC culture and differentiation, the same media employed for 2D culture and differentiation were used, however, washing of printed constructs was performed immediately after printing and gelation (see Bioprinting below). Washing was performed by rinsing constructs for 1 min three times in 37°C culture medium followed by two 10 min washes and 1 h incubation in media before ongoing culture with 100 U/mL penicillin and 100 µg/mL streptomycin (Life Technologies) under 5% CO₂ at 37°C.
Cells used in this study are approved for use by the University of Wollongong’s Human Research Ethics Committee (HE14/049), and regularly tested and maintained mycoplasma free.

4.2.2 Bioink preparation

The detailed information of bioink synthesis has been described in the Section 2.3.1. Here, we used the optimal material which was described in the Section 3. The 1.5% w/v of Ag solution was prepared in PBS; by heating in a microwave oven, with agitation every 5 sec. Al was added to give 5% w/v and stirred at 60 °C for 30 min. Finally, CMC was added to give 3.5 or 5% w/v and stirred at 60 °C for 1 h. The final solutions were subsequently cooled to RT, in readiness for combining with human NSCs and direct-write printing.

4.2.3 Bioprinting for human NSCs

Samples were extrusion printed into a cubic construct (10mm x 10mm x 10mm) using a 3D-Bioplotter® System (EnvisionTEC GmbH). hNSC-laden bioink samples comprised 5x10^6 cells suspended in 0.5 mL bioink. Samples were loaded into a 55cc barrel (Nordson Australia Pty Ltd), centrifuged at 1000rpm and 15 °C for 1 min to remove air bubbles, placed in the printing magazine, and fitted with a 200 µm printing nozzle (Nordson Australia Pty Ltd). Blender™ open source software was employed to design the scaffold and translated into numerical code for printing onto autoclaved glass slides at 15 °C. The applied pressure for optimal bioink was 1.5-2.0 bar. Following printing, scaffolds were immersed in 2% w/v calcium chloride for 10 min for crosslinking[10].

4.2.4 Mechanical measurement of bioink

Variations of bioink in extrusion force were measured during sample deposition in real-time. Samples were loaded into a syringe with the plunger coupled to the upper clamp of an EZ-S mechanical tester (Shimadzu). Measurements were performed in compression mode using a
10 N load cell, with a constant strain applied at 0.2 mm s\(^{-1}\), and recording the force over time. Distilled water was used as a control.

Indentation modulus (E\text{Ind}) was also evaluated using an EZ-S mechanical tester but a flat stainless-steel indenter (1 mm in diameter) with a 2 N load cell was used to indent the samples at a rate of 0.1 mm/min. Again, three different samples were tested at a minimum of four different locations per sample.

### 4.2.5 Diffusion studies

Diffusion of solute into the gels was measured similarly to the previously described method [8, 11] as detailed described in Section 2.6.5. Briefly, three cylindrical hydrogels (n=3) of 2 cm x 0.35 cm diameter were immersed in PBS containing fluorescently labelled bovine serum albumin (FITC-BSA, 250 µg/mL, Sigma) and maintained at a constant temperature of 37 °C in a shaking water bath. Protein uptake was determined by sampling the solution and measuring the loss of protein over time until it reached equilibrium using a micro-plate reader (Fluostar Omega, BMG Labtech). Concentrations of BSA were calculated from a standard curve. Diffusion coefficients of FITC-BSA in the gels were calculated using a nonlinear regression method and modelled using the finite element method (COMSOL 4.2)[11-13].

### 4.2.6 Live/Dead human NSC analysis

Calcein AM (5 µg/mL) and propidium iodide (PI, 5 µg/mL) were used to identify live and dead cells respectively, according to Section 2.8.1. Briefly, hNSC-laden constructs were incubated with Calcein AM at 37 °C for 10 min, followed by a media change, incubation with PI for 1 min, and a further media change. A confocal microscope (Leica TSC SP5 II) was used for image acquisition, with images from a minimum of five optical planes per construct merged (to capture the maximal projection of whole cell aggregates) for analysis using Fiji (Image J) software. Three independent samples were evaluated for each gel composition. Depth coding of cells shown in Figure 4-1 was performed using the 3D Projection Tool in
Leica Application Suite X (LAS X) software (Leica).

**Figure 4-1** The distribution and the thickness measurement of the printed human NSC constructs.

Assessment by confocal microscopy of human NSCs encapsulated within an optimal 5% w/v Al, 5% w/v CMC and 1.5% Ag gel construct at day 7 after printing, with the left panel depicting the centre (star) and edge of a construct at a single optical plane, middle panel showing the mid-region of a construct at a single optical plane, and right panel showing depth coding of cells along the Z-axis (0 – 369 µm; ie. different colours represent different planes along the Z-axis).

### 4.2.7 Human NSC proliferation analysis

PrestoBlue™ cell viability reagent was used for human NSC proliferation studies, according to the manufacturer’s instructions. Briefly, at each time point measured, three hNSC-laden constructs were incubated with the reagent in culture medium for 1 hr at 37 °C. Following incubation, for each sample, 100 µl supernatant was transferred to a well of a 96-well plate and screened by a microplate reader (POLARstar Omega) to read fluorescence intensity. After processing, constructs were rinsed in culture medium and returned to culture, with the
process repeated for each time point until the study was completed.

4.2.8 Immunocytochemistry

The general method has been described in Section 2.8.3. Particularly, samples were fixed with 3.7 % PFA solution in PBS at RT for 30 min, rinsed in PBS, and then blocked and permeabilized overnight at 37 °C with 5% (v/v) donkey serum in PBS containing 0.3% (v/v) Triton X-100 (Sigma). Samples were subsequently incubated with fluorescence conjugated antibodies GFAP (mouse, 1:100; Cell signalling), SOX2 (rabbit, 1:100; Cell signalling), vimentin (rabbit, 1:200; Cell signalling), OLIGO2 (mouse, 1:100; Millipore), KI67 (mouse, 1:200; Invitrogen), TUJ1 (mouse, 1:100; Abcam) and nestin (mouse, 1:100; Invitrogen), or unconjugated primary antibodies synaptophysin (rabbit, 1:200; Millipore), GABA (rabbit, 1:200; Sigma) and GAD (rabbit, 1:500; Millipore) at 4°C overnight. On the second day, samples were rinsed with 0.1% Triton X-100 in PBS three times, and samples with unconjugated primary antibody were incubated with Alexa Fluor tagged secondary antibody (1:1000; Invitrogen) for 1 hr at 37 °C. Nuclei were visualised with 4',6-diamidino-2-phenylindole (DAPI, 10 μg/mL) at RT for 10 min and antifade reagent (Invitrogen) was employed to preserve the fluorescence signal. Samples were mounted onto glass coverslips using Aquamount (ThermoScientific) and imaged on a confocal microscope (Leica TSC SP5 II). Images were collected and analysed using Leica Application Suite AF (LAS AF) software (Leica).

4.2.9 RT-qPCR of 2D and 3D samples

The general method of RT-qPCR has been described in Section 2.8.5. Particularly, gel-encapsulated cells were extracted for subsequent RNA isolation by treatment with 0.05 M EDTA for 10 min to dissolve the gel [14]. After treatment, samples were centrifuged at 600 g for 5 min to collect the cells. For primer sequences, see Table 4-1.
<table>
<thead>
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<th>Forward</th>
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<th>Length (bp)</th>
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<tr>
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<tr>
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<tr>
<td><em>β-Actin</em></td>
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<td>CACACGCAGCTCATGTG</td>
<td>103</td>
</tr>
</tbody>
</table>

4.2.10 Calcium imaging

For calcium imaging, the general method has been described in Section 2.8.4. Here, 3D samples were loaded with 2 μM Fluo-4 in fresh culture medium, incubated for 30 min at 37 °C, and washed with Tyrode’s solution (5 mM KCl, 129 mM NaCl, 2 mM CaCl2, 1 mM MgCl2, 30 mM D-Glucose and 25 mM HEPES, Ph 7.4) [15]. Samples were mounted on coverslips and imaging was performed at RT on a confocal microscope (Leica TSC SP5 II). LAS AF Lite software (Leica) was used to collect and quantify time-lapse excitation ratio images. GABA(A) receptor antagonist bicuculline (50 μM; Sigma) was added into Tyrode’s
solution for 3 min to induce intracellular calcium. Depth coding of cells was performed using the 3D Projection Tool in LAS AF software (Leica).

4.2.11 Statistical analyses

Statistical analyses were performed in OriginPro 2015 (Version b9.2.272) using one-way analysis of variance (ANOVA) with Bonferroni multiple comparison post hoc test or two-way ANOVA with Bonferroni post hoc test. Homogeneity of variance tests were performed to confirm that statistical assumptions were met for ANOVA. Statistical significance was set at $P < 0.05$.

4.3 Results and discussion

4.3.1 Printable properties of Al-CMC-Ag gel

To generate hNSC-laden gel constructs, an optimal printable bioink of 5% weight per volume (w/v) Al, 5% w/v CMC and 1.5% w/v Ag (Al-CMC-Ag) was developed, as described in Chapter 3. To further support the results, specification of the properties of the gels was attempted. Printability of the optimal bioink was supported by its uniform consistency, with minimal fluctuations in extrusion force (around 8.5 N) required for printing and indicative of homogeneity within the solution (Figure 4-2). As expected, lower viscosity water, as control, showed a similarly constant but lower magnitude (around 2 N) extrusion force profile (Figure 4-2). Ink gelation following ionic-crosslinking resulted in an initial compression modulus ($E_{\text{Comp}}$) of around 7.5 kilopascals (kPa), with an indentation modulus ($E_{\text{Ind}}$) of around 4.75 kPa. While subsequent temporal analysis of $E_{\text{Ind}}$ indicated decreasing stiffness of the gel, the rate of change diminished, attaining stabilisation at around 0.8 kPa by day 10 (Figure 4-3).
Figure 4-2 Consistency/homogeneity of the bioink.

The bioink Al-CMC-Ag (green line) consistency/homogeneity is demonstrated by the extrusion force required for printing. Water control (blue line) was employed for comparison.

Figure 4-3 Indentation modulus (EInd; blue bars) of Al-CMC-Ag over time.

The data are shown as (mean ± S.D.; n = 3) and % modulus (green dots) remained at a specified time point relative to the initial modulus at day 0 (EInd0).
4.3.2 BSA diffusion in Al-CMC-Ag gels

Intrinsic gel permeability was studied by measuring the uptake of bovine serum albumin (BSA) by 5% and 3.5% w/v CMC gels and applying a non-steady state diffusion model[11]. For both gels, BSA uptake reached equilibrium by 8 h (Figure 4-4A), also shown by finite element modelling (COMSOL; Figure 4-5). The diffusion coefficients were \(6.56 \times 10^{-7}\) and \(5.56 \times 10^{-7}\) cm\(^2\)/s for 5% and 3.5% w/v CMC gels respectively. The subsequent release appeared to be slower than the uptake (Figure 4-4B); these results indicated that nutrients, growth factor and cellular wastes can move in and out of the gels. Confocal microscopy imaging of FITC-BSA was performed at a single optical plane that was distant from the point of delivery, demonstrating diffusion throughout the 5% w/v CMC gels (Figure 4-6). The image-based analysis confirmed the rate of uptake and diffusion of BSA throughout the gel (Figure 4-7). The quantitative data show increasing fluorescence intensity from the point of delivery, supporting diffusion throughout the gel (Figure 4-8).
Figure 4-4 Diffusion of FITC-BSA from solution into and out of gels.

A) Uptake by diffusion of FITC-BSA from immersion solution into 5% and 3.5% w/v CMC gels. Diffusion of BSA from solution into submerged gels is indicated by decreasing measures of fluorescence in solution (data for a specific time point normalised against data for initial time point; Ct/C0). B) Release by diffusion of FITC-BSA from 5% and 3.5% w/v CMC gels into the bath solution, which is indicated by increasing measures of fluorescence in the bath solution.
<table>
<thead>
<tr>
<th></th>
<th>0 h</th>
<th>30 min</th>
<th>1 h</th>
<th>2 h</th>
<th>4 h</th>
<th>6 h</th>
<th>8 h</th>
<th>10 h</th>
<th>12 h</th>
<th>24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alg-CMC (3.5%)-Ag</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
<td><img src="image9.png" alt="Image" /></td>
<td><img src="image10.png" alt="Image" /></td>
</tr>
<tr>
<td>Alg-CMC (5%)-Ag</td>
<td><img src="image11.png" alt="Image" /></td>
<td><img src="image12.png" alt="Image" /></td>
<td><img src="image13.png" alt="Image" /></td>
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<td><img src="image15.png" alt="Image" /></td>
<td><img src="image16.png" alt="Image" /></td>
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<td><img src="image18.png" alt="Image" /></td>
<td><img src="image19.png" alt="Image" /></td>
<td><img src="image20.png" alt="Image" /></td>
</tr>
</tbody>
</table>

**Figure 4-5** Finite element model of BSA diffusion into Al-CMC-Ag containing 5% and 3.5% w/v CMC.

The model was simulated using COMSOL Multiphysics 5.0: 2D axisymmetric; 2 domains: gel (small/inset rectangle) and solution (large/enclosing rectangle).
**Figure 4-6** Schematic of set up for temporal analysis of FITC-BSA diffusion through Al-CMC-Ag gel.

FITC-BSA diffused from the top of the gel with 4mm thickness and imaged every 5 minutes.

**Figure 4-7** Assessment by confocal microscopy of FITC-BSA diffusion through optimal 5% w/v CMC gel.

The time course (0, 2h, 4h, 6h after loading FITC-BSA) of the fluorescent intensity at the bottom of the gel.
Photomicrographs and quantitative data show increasing fluorescence intensity at a single optical plane and distant from the point of delivery, supporting diffusion throughout the gel.

4.3.3 Bioprinting human NSC-laden Al-CMC-Ag gel constructs

Figure 4-9 illustrates the main processes of human NSC printing and *in situ* differentiation. Simply, the bioink without cells was first prepared from 5% w/v Al, 5% w/v CMC and 1.5% w/v Ag. The human NSCs were then mixed together with the hydrogels followed by direct printing on the 3D printer. There were two culture systems utilized, for self-renewal and differentiation respectively. Homogeneity was also reflected by uniform human NSC distribution and viability throughout the construct immediately following printing (Figure 4-10). Confocal microscopy revealed persistent homogenous live cell distribution throughout the constructs, with human NSCs visible as single cells on day 1 following printing, and aggregates of cells increasingly apparent thereafter (day 3 – day 11) (Figure 4-11). Further growth profiling of viable and dead cells over time within the gels demonstrated relatively
high (around 25%) cell death immediately after printing (day 0), with the proportion of dead human NSCs subsequently decreasing ($F(7, 30) = 14.10, P < 0.0001$) to being statistically significant on day 4 post-printing ($P < 0.001$), continuing to around 8% by day 6 (Figure 4-12). Live cell analysis supported cell proliferation ($F(9, 20) = 146.62, P < 0.0001$), reaching significantly different results on day 5 compared with day 1 after printing ($P < 0.0001$), before peaking at day 11 (Figure 4-13).
Figure 4-9 Schematic illustrating the major stages of the method for direct-write printing human NSCs with bioink for 3D culture and differentiation.
Figure 4-10 Printed hNSC-laden Al-CMC-Ag bioink.

Left) Printed gel scaffold comprising 5% w/v Al, 5% w/v CMC and 1.5% w/v Ag. Right) Live (Calcein AM) and dead (propidium iodide; PI) human NSC staining within printed Al-CMC-Ag gel scaffold.

Figure 4-11 Printed human NSCs within the Al-CMC-Ag gel construct showing grid/scaffold structure at specific time points.

Human NSCs are initially visible as single cells immediately following printing (day 1), with aggregates of cells increasingly apparent over time (day 3 – day 11).
Figure 4-12 Dead cell content versus time post printing.

Time course of dead human NSC content of Al-CMC-Ag gel from day 0 to day 7 after printing (mean ± S.D.; n = 3). One-way ANOVA with Bonferroni post hoc test. * P < 0.001 (day 4 vs day 0).

Figure 4-13 Cell proliferation in printed hydrogel.
Time course of live (PrestoBlue™ cell viability indicator) human NSC content of optimal 5% w/v CMC gel from day 1 to day 19 after printing (mean ± S.D.; n = 3). One-way ANOVA with Bonferroni post hoc test. * P < 0.0001 (day 5 vs day 1).

4.3.4 Characterization of encapsulated human NSCs

Immunophenotyping at three weeks post-printing demonstrated human NSCs expressed undifferentiated cell markers SOX2 (Figure 4-14A, C), vimentin (Figure 4-14A, D) and nestin (Figure 4-14B), as well as the nuclear proliferation marker KI67 (Figure 4-14B). Negligible levels of the differentiation and neuronal-specific cytoskeleton protein TUJ1 (Figure 4-14C), astrocyte marker glial fibrillary acidic protein (GFAP) and oligodendrocyte lineage transcription factor 2 (OLIGO2) were expressed (Figure 4-14D).
Figure 4-14 Immunophenotyping of human NSCs encapsulated within an Al-CMC-Ag gel construct 3 weeks after printing.

A) Human NSCs stained with DAPI colocalised with SOX2, and expressed vimentin. B) Cells also expressed nuclear proliferation marker KI67 and human NSC marker nestin. C) Human NSCs expressed negligible levels of differentiated neuron marker TUJ1. D) Human NSCs expressed negligible levels of differentiated astrocyte and oligodendroglial lineage markers GFAP and OLIGO2 respectively.
4.3.5 Characterisation of in situ differentiated human NSCs

Based on human NSC growth profiling consideration was given to exploring in situ differentiation by inducing human NSCs to functional neurons and neuroglia 10 days post-printing. Immunophenotyping 2 weeks after initiating differentiation revealed neurons had expressed TUJ1 (Figure 4-15A), GABAergic markers GABA and glutamic acid decarboxylase (GAD) (Figure 4-15B), with concomitant low SOX2 expression (Figure 4-15A). OLIGO2 and GFAP expression were mutually exclusive (Figure 4-15C), and presynaptic vesicle glycoprotein synaptophysin was apparent as small puncta often adjacent to cell bodies (Figure 4-15D). In addition, immunolabelling of TUJ1 3 weeks after differentiation confirmed persistent cell viability within constructs, with neuronal cell clusters interconnected by neurites (Figure 4-15E).

Gene expression analysis by reverse-transcription quantitative PCR (RT-qPCR) corroborated immunophenotyping by showing upregulation of pan-neuronal and neuroglial markers along with neuronal sub-type specific markers under differentiation conditions (Figure 4-16). RT-qPCR also included comparison between 3D and conventional planar human NSC culture and differentiation, with 3D differentiation predominantly associated with higher transcript levels for neuronal and neuroglial markers compared with 2D differentiation (Figure 4-16). Specifically, in situ 3D differentiation induced a higher expression of TUJI $(F(3, 4) = 1380.21)$, GFAP $(F(3, 4) = 55171.42)$, OLIGO2 $(F(3, 4) = 1667.92)$ and synaptophysin $(F(3, 4) = 315.75)$ mRNA compared with undifferentiated 3D human NSC constructs, and conventional 2D culture and differentiation (Figure 4-16). Interestingly, GFAP expression was markedly increased. Also, 3D gel-based differentiation accelerated upregulation of GABAergic neuronal marker GABA $(F(3, 4) = 1239.48)$, and to a lesser extent other GABAergic markers NKX2.1 $(F(3, 4) = 69.52)$ and MYST $(F(3, 4) = 2172.40)$, as well as transcripts relevant to other neuronal subtypes included, vesicular glutamate transporter
(VGLT; $F(3, 4) = 769.13$), serotonin transporter (SRT; $F(3, 4) = 315.75$) and serotonin neuronal maker PET1 ($F(3, 4) = 11.66$) (Figure 4-16).

The next step was the investigation of functional maturation of in situ differentiated human NSCs by measuring spontaneous and bicuculline-induced calcium response of neurons. Neurons displayed spontaneous calcium spikes (Figure 4-17A), and recurrent bursting activity was induced through disinhibition of cells by application of bicuculline, a GABA(A) receptor antagonist (Figure 4-17B).

A final assessment of neurons within the 3D construct was made by SEM, revealing cells with rounded soma and extensive neurite outgrowth (Figure 4-17C).
Figure 4-15 Immunophenotyping and gene expression of differentiated human NSCs encapsulated within an Al-CMC-Ag gel construct.
A) Cells (24 days post-printing, including 14 days differentiation) stained with DAPI and expressed neuronal marker TUJ1 with negligible levels of human NSC marker SOX2. B) Neurons expressing GABAergic neuron markers GABA and GAD. C) Gliogenesis within neural constructs supported by astrocyte and oligodendroglial lineage markers GFAP and OLIGO2 respectively. D) Synaptogenesis within neural constructs illustrated by presynaptic protein synaptophysin. E) Cells (31 days post-printing, including 21 days differentiation) stained with DAPI and expressed TUJ1, with cell clusters interconnected by neurites. The lower right panel shows depth coding of cells along the Z-axis (0 – 59 μm).

Figure 4-16 Comparative gene expression between conventional 2D and printed 3D human NSC culture and differentiation.

The culture duration was 3 weeks and differentiation duration was 5 days initial human NSC culture followed by 16 days of differentiation. Relative gene expression represents data normalized to β-actin and expressed relative to 2D human NSCs. Mean ± S.D.; n = 3. One-way ANOVA with Bonferroni post hoc test. *P < 0.05; **P < 0.01; ***P < 0.001.
**Figure 4-17** Functional maturation of in situ differentiated human NSCs in printed Al-CMC-Ag construct.

**A**) Time course of live calcium imaging of neurons within a 3D construct, with the lower left panel showing depth coding of cells along the Z-axis (0 – 169 µm; i.e. different colors represent different planes along the Z-axis), and average measurements of spontaneous activity for cells 1-3 of photomicrographs demonstrated by the corresponding plot. Arrowheads indicate active cells. **B**) Time course of live calcium imaging of neurons within a 3D construct, with the middle right panel showing depth coding of cells along the Z-axis (0 – 107 µm), and average measurements of bicuculline-induced calcium response for cells 1-2 of photomicrographs demonstrated by the corresponding plot. Arrowheads indicate active cells. **C**) SEM image showing a neuron inside a porous 3D construct, with an arrowhead and arrow indicating a rounded cell soma and extending neurite respectively.
4.4 Conclusion

3D extrusion bioprinting offers a versatile platform for fabricating human cell-based tissue constructs from novel clinically-relevant biomaterial-cell combinations. To date, several examples of cell printing have been described and include human dermal fibroblasts and umbilical vein endothelial cells (HUVECs) [16], hepatocarcinoma cells (HepG2)[4], adipose stem cells[5, 6], and mesenchymal stromal cells[6]. Non-human cell printing has included murine embryonic stem cells for \textit{in situ} embryoid body formation[7], and primary murine cortical neurons and myoblasts[8, 9].

Here we describe the first example of direct-write printing of hNSC-laden bioink to engineer a novel functional 3D nMTC. Co-printing of cells with the bioink provides an efficient, defined and simple approach to biomaterial-cell interfacing, with post-printing gelation resulting in cell encapsulation for \textit{in situ} human NSC expansion and differentiation. The 3D tissues generated are amenable to characterisation for studying neural development and function, including understanding how microenvironmental features affect cell and tissue phenotypes, and has the potential to be adapted to other stem cell types for generating neuronal and non-neuronal tissues \textit{in vitro}. Importantly, the bioink comprises widely available, inexpensive and well characterised components Al, CMC and Ag that have been optimally combined to form a printable, clinically-compatible gel. The consistency of the bioink solution underpins its reliability for printing constructs anew and constructs that are homogenous for uniform cell supportability. This is reflected by demonstrated homogenous human NSC distribution and viability throughout the construct following printing. Studies of modulus after gelation quantitate the mechanical stiffness and therefore construct integrity necessary for post-printing maintenance and handling, and cell support. Interestingly, the stiffness of the investigated gel is in the range of human brain tissue, with previous reports of \textit{in vivo} stiffness ranging from 0.5 - 14 kPa [16-19]. While temporal studies of indentation
modulus suggest an initial rapid decrease in stiffness, the diminishing rate of reduction combined with human NSC survival, proliferation and differentiation support enduring biocompatibility with lower gel moduli.

The mechanical properties of a gel, including the modulus and porosity of the matrix environment, affect cell behaviour such as proliferation and differentiation[20]. Thus, Al was initially chosen as the basis of the gel due to its recognised stability as a 3D structure, low toxicity, and cytocompatible gelation, whilst CMC sustained human NSC survival ostensibly by influencing gel porosity and permeability, and Ag provided requisite bioink viscosity for optimal Al-CMC printing. Other known properties conceivably beneficial to this approach include high moisture retention of CMC, and antimicrobial and low inflammatory responses of both Al and CMC, all features conducive to cell support and survival [21, 22]. Moreover, as a derivative of chitosan, CMC is deemed to have low to absent toxicity, no mutagenic effects, affects cellular expression of growth factors, and promotes cell adhesion, migration and proliferation [23, 24].

Characterisation of cells within this system supports human NSC self-renewal for several weeks following printing and therefore the ability to scale-up human NSC number in situ prior to differentiation. Importantly, human NSCs can be induced to functional neurons and supporting neuroglia, with gene expression analysis by RT-qPCR indicating differentiation of stem cells in the 3D constructs may be advantageous compared to conventional 2D platforms for accelerated neuronal, neuroglial and synapse formation. Interestingly, the highly expressed glial marker GFAP is consistent with its key role in central nervous system (CNS) processes including astrocyte-neuron interactions as well as cell-cell communication, with the latter extending to astrocyte mediated synapse formation and function [25-27]. The system may also bias neuronal differentiation to GABAergic lineage, making it attractive for inhibitory neuronal and tissue modelling. Notwithstanding, the occurrence of other neuronal
subtypes including glutamatergic and serotonergic, indicate the potential for more expansive modelling, with the possibility of enriching subtype neuronal expression through, for example, cytokine supplementation [28].

Finally, calcium imaging of functioning neurons within the 3D construct together with SEM imaging of neurons and neurites with complex 3D morphologies demonstrate platform utility for modelling human neural cell form and activity, and fabricating functional 3D human neural tissue. As such, the platform is amenable to translational drug-screening \textit{in vitro}, studying human neurodevelopment and disease, and possibly neural tissue engineering for CNS tissue replacement.
4.5 References


Chapter 5

3D Bioprinting human iPSC Constructs
for \textit{in situ} Cell Proliferation and
Successive Multi-lineage Differentiation

\begin{quote}
Most parts of this chapter present work that has appeared in the published article “3D bioprinting human induced pluripotent stem cell constructs for \textit{in situ} cell proliferation and successive multilineage differentiation” by Gu, Q., Tomaskovic-Crook, E., Wallace, G.G., and Crook, J.M. (2016). Adv Healthc Mater 5, 1429-1438. (cover)
\end{quote}
### 5 3D BIOPRINTING HUMAN iPSC CONSTRUCTS FOR IN SITU CELL PROLIFERATION AND SUCCESSIVE MULTI-LINEAGE DIFFERENTIATION

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#### 5.3 RESULTS AND DISCUSSION

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#### 5.4 CONCLUSION

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5 3D Bioprinting human iPSC constructs for in situ cell proliferation and successive multi-lineage differentiation

5.1 Introduction

Human iPSCs, like embryonic stem cells, have the ability to self-renew for large-scale expansion whilst maintaining the capacity to differentiate to all cell types (~200) of the human body [1-3]. These qualities together with the potential for autologous application make iPSCs compelling candidates for cell replacement therapies, tissue and organ engineering, and pharmacology and toxicology screening.

Since their discovery a decade ago, the development of culture protocols for human iPSCs has primarily focused on clinical-compliance [4], cell line stability [5], and efficiency of differentiation to desired cell lineages [6], all the while retaining conventional monolayer culture. Recent interest in recapitulating the 3D cytoarchitecture of native tissues in vitro to better simulate cell behavior in vivo is driving the application of 3D configured biomaterials to further advance stem cell (including iPSC) research and therapy [7]. By mimicking important features of a target tissue including the extracellular microenvironment, a 3D-biomaterial has the potential to instruct cell fate and function in a way not previously attainable with 2D culture [8]. Therefore, although still exploratory, it is envisioned that the synergism of stem-cell biology and 3D-biomaterial technology will be influential in iPSC-based research and translation.

Of the small number of 3D systems for iPSC culture reported to date, they all rely on the ability of iPSCs to self-organise by seeding onto or casting within supporting material such as conventional tumour-derived Matrigel® basement membrane preparation or more defined polymeric scaffolds [9-12]. An alternative, although previously untested, approach to bioengineering 3D iPSC constructs is to apply advanced 3D bioprinting for direct-write (or co-) printing of stem cells together with biomaterial to reproducibly generate tissue of a
desired architecture. 3D printing is one fast way to promote future regenerative medicine[13]. Co-printing represents a single-step approach to rapidly fabricate a 3D cell-material construct whereby iPSCs are immediately integrated with biomaterials by encapsulation for direct and complete contact with extracellular elements that more closely mimic the native cell microenvironment.

Here is described a body of work relating to iPSC printing following on from the PhD candidate’s previously published report of human neural stem cell (NSC) printing [14]. By utilizing the defined clinically-amenable polysaccharide-based bioink containing Al, CMC and Ag optimization has been achieved for extrusion printing of iPSCs (Figure 5-1A); able to be maintained as self-renewing stem cells within the printed construct, with cell proliferation enduring for at least 9 days post-printing (Figure 5-1B). Furthermore, stem cells can be differentiated in situ to self-assembling 3D cell aggregates called embryoid bodies with cells constituting all three primitive germ layers – mesoderm, ectoderm and endoderm (Figure 5-1C). Finally, following transition of the printed iPSC constructs to neural induction/differentiation media, more homogeneous neural tissues can be generated with neurons and supporting neuroglia (Figure 5-1D). Neurons are active, form synapses, participate in network activity and show migratory behaviour within a construct.

These findings affirm the efficacy of the PhD candidate’s previously described bioprinting platform for generating 3D tissues from human stem cells. Having now adapted the platform for iPSC printing and differentiation, its versatility has been verified for generating both neural and non-neural tissues including amenability to “notoriously difficult to culture” cell types such as human iPSCs [15, 16].
Figure 5-1 Schematic of the method for extrusion printing of iPSCs for 3D culture and differentiation.

A) Bioink is prepared by suspending iPSCs with clinically-amenable polysaccharides Al, CMC and Ag, followed by bioprinting and crosslinking for gelation. B) 3D iPSC-laden scaffolds are maintained in iPSC-culture medium for stem cell proliferation/self-renewal within the printed construct. C) iPSCs can be differentiated in situ to self-assembling 3D EBs comprising cells of all three primitive germ layers – mesoderm, ectoderm and endoderm, or D) more homogeneous neural tissues using neural induction/differentiation media. Neural constructs include functional neurons (including migrating) and supporting neuroglia.

5.2 Experimental

5.2.1 Human iPSC culture and differentiation

General 2D human iPSC culture has been detailed in Section 2.4.2. For 3D human iPSC culture and differentiation, the same media were used as 2D culture and differentiation. For
EB and neural differentiation, the media were replaced with corresponding media on the 5th day after printing. For 3D iPSC differentiation to neural lineage, neural induction medium (comprising DMEM/F12 supplemented with 1×N2 supplement, 2 µg/mL heparin (H3149, Sigma) and 1×MEM NEAA) was applied on the 3rd day after printing, and then 2-3 weeks later, the medium was changed to neuronal differentiation medium containing 2 parts DMEM F-12 : 1 part neurobasal supplemented with 2% StemPro, 0.5 % N2 and 50 ng/mL brain-derived neurotrophic factor (BDNF) for 3 weeks culture.

5.2.2 Bioink preparation and bioprinting

The bioink was prepared as previously described in section 2.3.1 [14]. Briefly, agarose was dissolved in PBS by heating in a microwave oven to give 1.5% (w/v), followed by addition of Al and CMC to give 5% (w/v). After 1 hr stirring at 60 °C, the final solution was cooled to RT and 4x10^7 iPSCs were added per 0.5 mL bioink.

Bioprinting platform and items have been described in Section 2.5. Bioink samples were loaded into the barrel and centrifuged at 300 g at 15 °C for 1 min to remove air bubbles, followed by printing onto autoclaved glass slides. After printing, the scaffolds were immersed in 2% w/v calcium chloride for 10 min for crosslinking. After gelation, the constructs were rinsed three times in culture medium followed by 2 x 10 min washes and incubation in fresh culture medium for 1 h to remove excess calcium ions.

5.2.3 SEM of printed 3D constructs for human iPSCs

The general process SEM has been described in Section 2.6.2. For SEM of printed constructs, samples were submerged in human iPSC culture media for 24 hr, freeze dried overnight using a Christ Alpha 2-4 LD Freeze Dryer, then coated with 20 nm gold using an Edwards sputter coater, and kept desiccated until analysed. SEM was performed using a JSM-7500FA LV Scanning Electron Microscope. For studies of internal structure, samples were fixed with 3.7% paraformaldehyde (PFA, Fluka) for 30 min, immersed in liquid nitrogen for 60 seconds, and
then freeze-fractured using a cold razor blade. The fractured samples were immediately observed on the JSM-6490 LV Scanning Electron Microscope.

5.2.4 Live/dead iPSC analysis

The method has been described in section 2.8.1. Briefly, printed human iPSC constructs were incubated at 37 °C with Calcein AM for 10 min, followed by PI for 1 min. A Leica TSC SP5 II confocal microscope was used for image acquisition, with images from a minimum of five optical planes per construct merged (to capture the maximal projection of whole cell aggregates) for analysis using Fiji (Image J) software. Three independent samples were evaluated for each gel composition. Depth coding of constructs was performed using the 3D Projection Tool in Leica Application Suite X (LAS X) software (Leica).

5.2.5 Immunocytochemistry

The general process of staining has been described in Section 2.8.3. Particularly, the 2D and 3D human iPSC samples were fixed with 3.7 % PFA in PBS for 30 min at RT. Samples were then blocked and permeabilized overnight with 5% (v/v) donkey serum in PBS containing 0.3% (v/v) Triton X-100 (Sigma) at 4 °C. Samples were subsequently incubated with primary antibodies against OCT4 (mouse, 1:200, STEMCELL Technologies), SSEA4 (mouse, 1:200, STEMCELL Technologies), TRA-1-60 (mouse, 1:200, STEMCELL Technologies), PAX6 (rabbit, 1:100, Sigma), nestin (mouse, 1:100; Invitrogen), synaptophysin (rabbit, 1:200; Millipore), TUJ1 (Chicken, 1:200, Millipore) and GABA (rabbit, 1:200; Sigma) or fluorescence conjugated antibodies GFAP (mouse, 1:100; Cell signalling), MAP2 (mouse, 1:100, Millipore), SOX2 (rabbit, 1:100; Cell Signalling) at 4 °C overnight. On the second day, samples were rinsed with 0.1% Triton X-100 in PBS three times and samples with unconjugated primary antibody were incubated with Alexa Fluor tagged secondary antibody (1:1000; Invitrogen) for 1 hr at 37 °C. Nuclei were labelled with 4',6-diamidino-2-phenylindole (DAPI, 10 μg/mL) at RT for 10 min and antifade reagent (Invitrogen) was
employed to preserve fluorescence signal. Samples were mounted onto glass coverslips using Aquamount (ThermoScientific) and imaged with a Leica TSC SP5 II confocal microscope. Images were collected and analysed using Leica Application Suite AF (LAS AF) software (Leica).

5.2.6 Human iPSC proliferation analysis

PrestoBlue™ cell viability reagent was used for iPSC proliferation studies, according to the Section 2.8.2. Briefly, at each time point measured, three cell-laden constructs were incubated with the reagent in culture medium for 1 hr at 37 °C. For each construct, 100 µl supernatant was transferred to a well of a 96-well plate and screened by a microplate reader (POLARstar Omega) to read fluorescence intensity. Constructs were subsequently rinsed in culture medium and returned to culture, with the process repeated for each time point until the study was completed.

5.2.7 RT-qPCR of 2D and 3D samples

General RT-qPCR process has been demonstrated in Section 2.8.5. For RNA isolation, conventional 2D cultured iPSCs, EBs and neural cells were treated with Trizol™ Reagent. 3D gel-encapsulated cells were first treated with 0.05 M disodium EDTA for 10 min, fluxed with a pipette, followed by centrifugation at 600 g for 5 min. The left processes were similar with Section 2.8.5 and not described again. Primer sequence information is provided in Table 5-1.

Table 5-1 Primers used for RT-qPCR in iPSC printing

<table>
<thead>
<tr>
<th>Name</th>
<th>Forward</th>
<th>Reverse</th>
<th>Length (bp)</th>
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<tr>
<td>OCT4</td>
<td>CAATTTGCCAAGCTCCTGA</td>
<td>CGTTTGGCTGAATACCTTCC</td>
<td>105</td>
</tr>
<tr>
<td>SOX2</td>
<td>TGCTGCCTCTTTAAGACTAGGAC</td>
<td>GCCGCCGATGATTGTATTTA</td>
<td>117</td>
</tr>
<tr>
<td>NANO2</td>
<td>TACCTCAGCCTCCAGCAGAT</td>
<td>TCGTCACACCATTGCTATT</td>
<td>146</td>
</tr>
<tr>
<td>TDGF1</td>
<td>CTTCAAGAGATGACAGCATATTGG</td>
<td>CAGCAGGTTCTGTATGCTCCT114</td>
<td>114</td>
</tr>
</tbody>
</table>
5.2.8 Flow cytometry

3D samples were first extracted from the 3D structures described above. 2D and 3D cultured iPSCs were initially digested in 0.02% EDTA for 5 min and 10 min respectively, triturated, and passed through a 40 µm sieve to generate single cell preparations. After trituration, single cells were pelleted followed by centrifugation at 300 g for 5 min and fixed with 3.7% paraformaldehyde solution in PBS on ice for 10 min. After 2 washes in PBS/0.1% (v/v) Triton X-100, cells were resuspended in blocking buffer (5% Goat Serum plus 0.3% Triton-x-100 in PBS) and placed on ice for 30 min. Cells were then incubated with primary antibodies for OCT4, SSEA4 and TRA-1-60 described above and SOX2 (rabbit, 1:200; STEMCELL Technologies), and diluted in wash buffer on ice for 30 min. Following a further 2-3 washes, secondary antibodies conjugated with Alexa Fluor tagged secondary antibody
(1:1000; Invitrogen) and diluted in blocking buffer were applied for 30 min on ice in the dark. Cells were then washed again before being resuspended in 2% FBS/PBS and analysed by a BD Accuri C6 system (BD Biosciences).

5.2.9 Calcium imaging

The whole process has been detailed in Section 4.2.10.

5.2.10 Statistical Analyses

Statistical analyses were performed in OriginPro 2015 (Version b9.2.272) using one-way analysis of variance (ANOVA) with Bonferroni multiple comparison post hoc test or two-way ANOVA with Bonferroni post hoc test. Homogeneity of variance tests were performed to confirm that statistical assumptions were met for ANOVA. Statistical significance was set at $P < 0.05$.

5.3 Results and discussion

5.3.1 iPSC bioprinting, survival and in situ proliferation

There was homogenous distribution of human iPSCs after 3D printing (Figure 5-2). Extrusion printing of optimal iPSC-laden bioink resulted in the generation of scaffolds containing uniformly distributed stem cells throughout (Figure 5-2A, B and D). Encapsulated cells were viable with negligible cell death apparent immediately after ink gelation by ionic-crosslinking, persisting through extended culture in excess of 7 days (Figure 5-2). iPSCs showed characteristic pluripotent cell morphology, similar to embryonic stem cells (ESCs), being round in shape with large nuclei and sparse cytoplasm. During the course of maintaining constructs for stem cell expansion, single iPSCs proliferated to form aggregates of cells culminating in large spheroids by day 7. Spheroids could be clearly seen abutting the lumen of scaffolds and dispersed throughout the gel. The phenomenon of spheroid formation is consistent with colony formation during conventional 2D culture, but with spheroids reflecting well-defined clusters of tightly packed cells within a 3D system as compared to
classical sharp-edged, flat, tightly packed colonies.[16] iPSC-growth profiling showed cell proliferation increased from the time of printing and peaking at day 9 (Figure 5-3).

**Figure 5-2** Printed human iPSCs pattern in the hydrogels.

Live (Calcein AM) and dead (Propidium iodide; PI) iPSC staining within a printed construct at days 1, 3 and 7 post-printing. Initially encapsulated iPSCs are visible as evenly distributed single cells, with aggregates of cells increasingly apparent over time. By day 7 cell aggregates appear as large spheroids, abutting the lumen of scaffolds, though dispersed throughout the gel.

**Figure 5-3** Cell proliferation in the printed hydrogels.
Time course of live (PrestoBlue cell viability indicator) iPSC content of gel constructs up to day 11 after printing (mean ± S.D.; \(n = 3\)). One-way ANOVA with Bonferroni post hoc test. *\(P < 0.0001\) (day 7 vs day 1).

5.3.2 Pluripotency of human iPSCs in 3D printed constructs

Different methods were used to determine the pluripotency of human iPSCs in the 3D gels. Flow cytometry of iPSCs extracted from printed constructs after 10 days culture revealed ubiquitous expression of pluripotency cell markers OCT4, SOX2, TRA-1-60 and SSEA4, consistent with a persistent pluripotent stem cell state (Figure 5-4A) compared with conventional 2D cultured human iPSCs (Figure 5-5). Cell pluripotency was also confirmed by formation of prototypical iPSC-colonies from similarly extracted spheroids that were sub-cultured by the conventional 2D-planar method (Figure 5-4B). Moreover, immunophenotyping with confocal microscopy showed that iPSC-spheroids within constructs again expressed OCT4, SOX2, TRA-1-60, and SSEA4, which corresponded to flow cytometry analysis (Figure 5-6).

Figure 5-4 Pluripotency of human iPSCs in 3D printed gels.
A) Flow cytometry of iPSCs extracted from 3D constructs 10 days post-printing (See also Figure S1). B) Following extraction of iPSC-spheroids from 3D printed constructs (ie. 11 days post-printing) they could be recovered for conventional planar sub-culture forming classical iPSC-colonies on Matrigel® basement membrane matrix. Scale bars as indicated.

**Figure 5-5** Flow cytometry of conventional 2D cultured human iPSCs.
Cells were maintained in 5% CO₂ at 37°C, mTeSR™1 on Matrigel® basement membrane matrix in 6-well plates.
Figure 5-6 Immunostaining of printed human iPSCs in 3D.

Immunophenotyping of 3D bioprinted human iPSCs 10 days post-printing. iPSCs formed spheroids and stained with DAPI colocalized with pluripotency markers A) OCT4 and B) SOX2.
SOX2, and expressed C) SSEA4 and D) TRA-1-60. Pseudocoloured images as indicated by colour of text. Scale bars as indicated.

5.3.3 In situ differentiation of iPSCs to EBs comprising cells of three germ lineages – endoderm, ectoderm, and mesoderm

The differentiation potential of printed constructs was initially investigated by directing 3D bioprinted iPSCs to form EBs within constructs. EBs are 3D cell aggregates, which mimic many of the hallmarks of embryonic development. As EBs develop, differentiated cell phenotypes of all three germ lineages arise.[17] Therefore in addition to demonstrating multi-lineage cell and tissue formation, the induction of EBs represents another (ie. functional) test of pluriotency.

EBs formed within 3D constructs following modification of the iPSC culture media to basic fibroblast growth factor (bFGF) -free medium 5 days post-printing (Figure 5-7A). They displayed archetypal morphology, with radiating and retracting projections (Figure 5-7B). The observed projections are consistent with EBs often exhibiting tissue-like structures, such as the patterning of neurite extensions indicative of neuron organization.[18] Notwithstanding the evidence for neural cell lineage, assessment of gene expression of extracted EBs by reverse-transcription quantitative PCR (RT-qPCR) confirmed downregulation of pluripotency markers OCT4, NANOG, TDGF1, and UTF1 compared with undifferentiated EB-controls, with increased expression of endodermal (H19 and PSXI), mesodermal (HAND1 and IGF2) and ectodermal (NESTIN and TUJ-1) markers confirming iPSC differentiation to all germ lineages (Figure 5-7A, Figure 5-8). Taken together, these data substantiate the potential to form multiple cell and tissue types within and from the bioprinted constructs. Secondarily, the results are consistent with having maintained iPSC status for the period of preserving constructs in culture after printing, prior to differentiation.
Figure 5-7 In situ formation of EBs from 3D bioprinted human iPSCs 15 days post-printing (including 10 days iPSC differentiation).

A) EB formation within 3D constructs (arrowheads) which could be extracted for further RT-qPCR. B) EBs formed within 3D constructs, displayed typical morphology, with elongated cell projections resembling neurite extensions radiating out and retracting over time (arrowheads). Scale bars as indicated.
Figure 5-8 Gene expression relative to conventional 2D culture cells.

Comparative gene expression (OCT4, NANOG, TDGF1 and UTF1: pluripotency markers; H19 and PDX1: endodermal markers; Hand-1 and IGF2: mesodermal markers; NESTIN and TUJ-1: ectodermal markers) between conventional 2D and 3D iPSCs and EBs. Relative gene expression represents data normalized to β-actin and expressed relative to 2D iPSCs. Mean ± S.D.; n = 3. One-way Anova with Bonferroni post hoc test. *P< 0.05; ** P< 0.01; *** P< 0.001.
5.3.4 Directed differentiation of 3D bioprinted iPSC constructs to neural tissues

While without specific medium supplements pluripotent stem cells have a tendency to differentiate to derivatives of the three germ lineages, alternative media compositions (including the use of defined growth factor additives) can promote differentiation toward one or another lineage. [6] Given the PhD candidate’s earlier work on generating neural tissues using bioprinted NSCs, attention was drawn towards similarly generating neural tissue from the 3D bioprinted iPSCs.

Differentiation involved an intermediate progenitor phase by culturing constructs in neural induction medium for 2-3 weeks from the third day after printing, followed by differentiation (using further modified medium including brain-derived neurotrophic factor; BDNF) into mature cells with phenotypes representative of different neuronal subtypes and supporting neuroglia. Consistent with forming iPSC-derived neural progenitors, immunophenotyping of constructs following 17 days of neural induction (20 days post-printing) revealed cells expressing neural progenitor markers PAX6 and NESTIN (Figure 5-9). Subsequent analysis of further differentiated constructs (> 30 days of neural induction) confirmed maturation to tissues comprising cells expressing pan-neuronal markers microtubule-associated protein 2 (MAP2; Figure 5-10A and D) and class III beta-tubulin protein (TUJ1; Figure 5-10C), as well as radial glial and astrocyte marker glial fibrillary acid protein (GFAP; Figure 5-10B and D) and presynaptic vesicle glycoprotein synaptophysin (Figure 5-10C). MAP2-expressing neurons often abutted the neuroglia (Figure 6D) and synaptophysin colocalised to neurites and neuronal cell soma, including longer neurite projections between neuronal cell clusters (Figure 5-10C).

Further immunocytochemistry together with RT-qPCR substantiated discrete neural cell subtypes including gamma-aminobutyric acid (GABA) expressing neurons (Figure 6E), corroborated the findings by transcript for NKX2.1, as well as serotonergic marker PETI and
oligodendrocyte lineage transcription factor 2 (OLIG2) (Figure 5-11). RT-qPCR also confirmed increased transcript for neuronal marker NESTIN, TUJ1 and GFAP, with highest levels of expression for 3D differentiated iPSCs compared to undifferentiated controls and 2D differentiated iPSCs, as well as concurrent downregulation of pluripotency markers OCT4, NANOG, and SOX2 (Figure 5-11). Finally, consistent with the presence of GABAergic neurons, neurons displayed recurrent increases in extracellular calcium concentration in response to GABA receptor-A antagonist bicuculline (Figure 5-12A). Functionality was also supported by neuronal cell migration within constructs, including characteristic long and dynamic leading processes (Figure 5-12B). Migrating neurons generally exhibit a leading process, with some being branched and others single. [19] The example presently shown is a neuron with a “relatively unbranched process” that appears to retract and extend and a tip that clearly moves forward as the cell traverses the construct. Taken together, the above findings verify the ability to differentiate iPSCs within the bioprinted constructs and in particular their conversion to functional neural cells for 3D neural tissue formation.

Figure 5-9 Characterization of neural precursors differentiated from human iPSCs in 3D constructs.
Immunophenotyping of 3D bioprinted human iPSCs 20 days post-printing including 17 days of neural induction. A) Cells stained with DAPI and expressed neural progenitor markers PAX6 and nestin. B) Depth coding of cells along the Z-axis of a 3D printed construct (0 – 107 μm). Pseudocoloured images as indicated by colour of text. White dashed lines of DAPI and PAX6 labelling images outline the lumen of the printed scaffold/construct. Scale bars as indicated.
Figure 5-10 Immunophenotyping of 3D bioprinted human iPSCs 40 days post-printing including 30-37 days of neural induction.
A) Cells stained with DAPI and expressed pan-neuronal marker MAP2 revealing neural processes extending throughout constructs, as well as B) radial glia and astrocyte marker GFAP; also shown, depth coding of cells along the Z-axis 0 – 63 µm and 0 – 56 µm respectively. C) Cells stained with DAPI and pan neuronal-marker TUJ1. Synaptophysin colocalised with TUJ1-labelled processes extending between neuronal cell clusters. D) MAP2 expressing neurons with neurites abutting and partially colocalised with GFAP expressing glial cells. E) GABAergic subtype neurons expressing GABA. Pseudocoloured images as indicated by colour of text. Scale bars as indicated.
Figure 5-11 Comparative gene expression between conventional 2D and 3D (bioprinted) human iPSC and neural derivative cultures.

(Pluripotency: OCT4, Nanog, SOX2; Neural: nestin, TUJ1, GFAP, GABA, NKX2.1, PET1, OLIG2). Mean ± S.D.; n = 3. One-way ANOVA with Bonferroni post hoc test. *P < 0.05; **P < 0.01; ***P < 0.001.
Figure 5-12 Live cell imaging of calcium flux and cell migration showing functional neurons forming networks within 3D structures 40 days after printing iPSCs including 37 days of neural induction.

A) Time course of bicuculline-induced calcium flux for individual neurons 1-2 within a 3D construct. The photomicrographs and corresponding line-plots show active cells (arrowheads) and average measurements of calcium flux respectively. Also shown, is the depth coding of cells along the Z-axis of the 3D printed construct (0 – 137 µm). B) Live cell imaging demonstrating neuronal cell migration within a 3D construct (large arrows: cell soma; small
arrows: leading process). Pseudocoloured images as indicated by colour of text. Scale bars as indicated.

5.3.5 3D printing growth factors to drive human iPSCs growth and penetration

To prove cell growth and cell movement in the 3D hydrogel, the PhD candidate’s previous data have shown that cells could expand with immuno-staining. Until now, there is still no report indicating live cell growth or recording the details of cells in 3D. The samples were mounted on the Leica Confocal Microscope platform with the live cell culture system after printing and differentiation of iPSCs. A few neuronal-like axons elongated from the EBs and the single cell migration in 3D system (Figure 5-7B, Figure 5-12B) were observed. Tissue function is dependent on the cell gradients, and growth factors are well known to regulate cell behaviours. The bFGF nano-particles (Figure 5-14A) were selected to be printed in parallel alongside human iPSCs printing. There were two barrels used, which contain two different bioinks respectively (one ink was the gel with human iPSCs and the other one just containing the beads with or without bFGF) as Figure 5-13 shows. Cell growth analysis supported the higher cell proliferation in comparison with the blank beads (Figure 5-14B). There were also obvious colonies and migration observed in the group of bFGF beads (Figure 5-14C).

Figure 5-13 Constructs printed from two different bioinks.
The scheme of 3D printing beads (A) and the samples printed from two different materials (B). There were two types of beads chosen: the beads with bFGF and the beads without bFGF, which are commercially available.

**Figure 5-14** Cell growth and migration under 3D printed bFGF influence.

A) bFGF nano beads. B) Live/dead images of the group of blank beads and bFGF beads at day 3 and day 10 during cell growth in the printed pattern. The dash lines demonstrate the boundary of the two inks. C) Time course analysis of the growth rate of blank group and bFGF bead group.

**5.4 Conclusion**

This work represents a first demonstration of bioprinting iPSCs for ensuing culture and expansion within a printed construct. In addition, the results demonstrate the ability to sequentially differentiate printed iPSCs *in situ* to multiple lineages representative of all three germ layers – mesoderm, ectoderm and endoderm, as well as form more homogeneous neural tissues. To the best of the PhD candidate’s knowledge, the only other example of bioprinting
pluripotent stem cells, and more specifically iPSCs, involved printing with cell culture media for subsequent immediate testing of post-printing cell viability and pluripotency.[20] iPSCs were neither cultured nor differentiated following printing, and supporting biomaterial was not employed in the bioink. Accordingly, this work has overcome reputed difficulties with maintaining and differentiating iPSCs, in spite of printing, by using a bioink with well-characterised and inexpensive components, having optimal viscosity for initial cell support during printing, and continuing support after printing and gelation. Al, CMC and Ag are widely available and used for clinical purposes, each having inherent qualities beneficial to the bioprinting process and/or cell survival. [14, 21, 22] While brief details of their individual and combined properties have been previously given, Al enables cytocompatible gelation using calcium chloride, with CMC providing favorable porosity and related permeability, and Ag is necessary for optimum bioink viscosity. [14] Moreover, CMC has other known beneficial properties such as high moisture retention, low inflammatory, toxicity and antimicrobial responses, and promotion of cell adhesion, migration and proliferation.[22-24] The stem cells in 3D conditions maintain good migration ability and the involvement of specific factor/condition could enhance the effect.

In summary, the ability to 3D print human iPSCs to then expand and generate cells of different lineages provides an unprecedented opportunity to form different, authentic and renewable body tissues. To this end, the present body of work represents an important first step, with further refinement of the method expected to benefit enhancement of tissue identity, architecture and function to better model development and diseases; for pharmaceuticals screening and assessing in vivo function and safety in animal models towards transplantation therapies.
5.5 References


Organoids From Human Induced Pluripotent Stem Cells Using a Chemically Defined Hydrogel and Defined Cell Culture Medium, Stem cells translational medicine 5(7) (2016) 970-9.


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Chapter 6 Conductive Material

Development for Stem Cell Stimulation
6 CONDUCTIVE MATERIAL DEVELOPMENT FOR PLURIPOTENT STEM CELL STIMULATION

6.1 INTRODUCTION

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6 Conductive material development for pluripotent stem cell stimulation

6.1 Introduction

The previous work mainly described the development of printable biomaterials for stem cells and stem cell growth in the 3D printed structure. To improve the property of materials for pluripotent stem cells for future use, iPSC stimulation was investigated.

Pluripotent stem cells have attracted much interest because of their capability of differentiation into many cell types. Electric fields, generated from the difference between the interior and the exterior of a biological cell, contribute to embryonic development and direct cell-cell communication in the human body [1, 2]. Electrical stimulation of neural tissues, both peripheral and central, has been widely used to study and/or treat spinal cord injury, nerve injury and deep brain injury [3-5]. Conductive polymers (CPs), also known as electroactive polymers, were discovered several decades ago and it has been found that doping is essential for good conductivity of these polymer[6]. CPs are experiencing a rapid increase in interest as biomaterials used in tissue engineering applications. Conductive substrates are able to improve nerve cell activity and could also be used to support cardiac progenitor cell growth [7, 8]. In the body, some cell types including nerve and muscle cells were found to react to electrical signals and some biocompatible CPs have been developed to stimulate them [9, 10].

PPy is a type of conductive polymers which has been treated with different dopants including anionic dopant dodecylbenzenesulfonate (DBS), para-toluene sulfonate (pTS) and the sodium salt of chondroitin sulfate (CS) during the process of oxidative polymerization [11]. PPy has been used in various fields including fuel cells [12], stimulation systems [9], biosensors [13] and for drug delivery[14]; and has been proven to possess good chemical stability and good biocompatibility in vitro and in vivo [15, 16]. An increase in bioactivity has been observed which was due to the doping or conjugation of growth factors and bio-proteins [17, 18].
The strategy of electrical stimulation of stem cells offers advantages including proliferation and differentiation of neural stem cells [19], cardiac differentiation from mesenchymal stem cells (MSCs) [20] and neurite growth and neural differentiation [9]. There have been some reports of embryonic stem cell (ESC) stimulation. However, the cells stimulated were embryoid bodies which were generated from mouse and human ESCs [21, 22]. There were various differences between different cell types in terms of morphology, differentiation ability and genotype[23]. The role of stimulation in the culture of human pluripotent stem cells remains unclear. Here, human iPSCs were able to proliferate on a film of PPy-DBS substrate and then could be differentiated into neurons after the application of electrical fields. The 2D stimulation results contributed to the subsequent complex 3D stimulation.

6.2 Experimental

6.2.1 Preparation of polymer films

The detailed PPy-DBS film preparation process was described in Section 2.3.2. Briefly, Polymer electrosynthesis was performed in a three-electrode cell with the gold-coated mylar as the working electrode, a platinum mesh counter electrode (CE), and Ag|AgCl reference electrode (RE). The films were washed with ddH₂O and dried using N₂ gas after growth. Then, the custom modules were assembled with culture chamber (Nunc® Lab-Tek® Chamber Slide™) as in Figure 2-16 and then stored under sterile conditions until use.

6.2.2 Cyclic voltammetry

Cyclic voltammetry (CV) was performed with a CH Instruments 660D Electrochemical Workstation using a three-electrode cell with a 3 cm² PPy-DBS coated mylar working electrode, a platinum mesh counter electrode (CE) and an Ag|AgCl reference electrode (RE). A scan rate of 0.1 V/s over the potential range of – 0.7 to + 0.7 V was used.
6.2.3 Human iPSC culture and stimulation

Normal human iPSCs culture has been described in Section 2.4.2. The PPy-DBS films were coated with Matrigel® at 4°C overnight before use. The colonies were mechanically cut into smaller colonies then relocated onto PPy-DBS films in pre-warmed mTeSR™1 and cultured for 24h (this time was set as Day 1) before stimulation to allow colonies to adhere to the PPy-DBS substrates. Cells were stimulated for 8h per 24h until Day 4 using a current density of $\pm 0.1$ mA/cm² and then 8h per two days at $\pm 0.25$ mA/cm² to Day 10. The cells were stimulated at 250Hz using a biphasic waveform of 100 ms pulses with 20 ms interphase open circuit potential. In response to the current pulse applied, the voltage waveform across the electrode area was recorded and total impedance ($Z_t$) was calculated from maximum voltage ($V_t$) divided by the measured current output ($i$) ($Z_t = V_t/i$). The access resistance and polarization impedance were obtained from the initial voltage drop ($R_a = V_a/i$) and the remaining voltage drop ($Z_p = V_p/i$) respectively.

6.2.4 RT-qPCR of the stimulation samples

For RNA isolation, the cells were treated with TRIzol™ on the PPy-DBS film followed by precipitation of RNA using isopropanol. The other processes have been described in Section 2.8.5. CFX software was used to collect the data which were analysed according to delta-delta Ct method. Primer sequence information is provided in Table 6-1.

Table 6-1 Primers used for RT-qPCR in iPSC stimulation

<table>
<thead>
<tr>
<th>Name</th>
<th>Forward</th>
<th>Reverse</th>
<th>Length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OCT4</td>
<td>CAATTTCGCAAGCTCCTGA</td>
<td>CGTTTGCTGAATACCTTCC</td>
<td>105</td>
</tr>
<tr>
<td>NANOG</td>
<td>TACCTCAGCCTCCAGCAGAT</td>
<td>TGCUGCACACCATTGCTATT</td>
<td>146</td>
</tr>
<tr>
<td>H19</td>
<td>GCAAGAAGCGGCTGTGTTT</td>
<td>GCTGGGATGCACCATTCTCTT</td>
<td>105</td>
</tr>
<tr>
<td>HAND1</td>
<td>AAGCGGAAAAGGGAGCTG</td>
<td>ACTCCAGCGCCCAGACTT</td>
<td>112</td>
</tr>
</tbody>
</table>
NESTIN GCCCTGACCACCTCCAGTTTA GGAGTCTGGATTTTCTTCC 200
PDX1 CTTTTCCATGGATGAAGTC GGAACCTCTTCTCCAGCTCTA 145
IGF2 CTGTITCCGCAGCTGTGAC GGGGTATCTGGGGAAGTTGT 118
TUJ1 ACACAGGGTGACACAGGGTTA GTTCCAGGTCCACCAGAATG 167
GFAP ATCAACTCACCGCACAACA CTTCATCTGCTTCTGTCTATA 153
OLIG2 TTGCTCCTCTTCCCTCCTT GGCTTCAACTAACTTGTG 129
GABA GTCCAGGTCTGTGACTGTCTT CTTCAGGGTTTACACCTCTTC 197
NXY2.1 CTTTGCTATACGGTCGGA GTGGATGGTGTCTGTGT 103
PET1 CGTCTTCTCCTCCTTGTCC CTCTACGAGGTACATAG 177
β-Actin AGGCATCCTCACCTGAAGTA CACACGCAGCTCATTGTA 103

6.2.5 Immunocytochemistry

The general method has been described in Section 2.8.3. Particularly, the stimulated and control samples were fixed with 3.7 % PFA in PBS for 10 min at RT. Samples were then permeabilized and blocked in 0.3% (v/v) Triton X-100 mixed in PBS containing 5% (v/v) donkey serum. The samples were subsequently incubated with primary antibodies for OCT4 (mouse, 1:200), SOX2 (rabbit, 1:200), SSEA4 (mouse, 1:200), TUJ1 (Chicken, 1:200) or fluorescence conjugated antibodies GFAP (mouse, 1:100) and VIMENTIN (rabbit, 1:200) at 4 °C overnight. Subsequently, the cells were rinsed with 0.1% Triton X-100 in PBS three times and then were incubated with Alexa Fluor tagged secondary antibody (1:1000) for the samples with unconjugated primary antibody incubation for 1 hr at 37 °C. Nuclei were stained with DAPI (10 μg/mL) at RT for 10 min, the chambers were carefully removed and antifade reagent was used to preserve the fluorescence density. Samples were covered with
glass coverslips and imaged on Leica confocal system (Leica TSC SP5 II) with software Leica Application Suite AF (LAS AF) for image collection.

6.2.6 Statistical Analyses

Statistical analyses were performed using OriginPro 2015 (Version b9.2.272) with application of a one-way analysis of variance (ANOVA) with Bonferroni multiple comparison post hoc test or two-way ANOVA with Bonferroni post hoc test. Homogeneity of variance tests was performed to confirm statistical assumptions were met for ANOVA. Statistical significance was set at $P < 0.05$.

6.3 Results and discussion

6.3.1 Human iPSC culture on PPy-DBS film

Figure 6-1 shows the method for human iPSC stimulation. The pyrrole with dopant DBS was initially polymerized on the gold film and then the custom devices were made (Figure 6-2). The cyclic voltammogram (CV) demonstrated the electroactivity of PPy-DBS with the typical oxidation and reduction processes (Figure 6-3). Three impedances of the substrate were calculated using Ohm's law and the values were monitored during the stimulation period (Figure 6-4), which consisted of total impedance ($Z_t = V_t/i$), access resistance ($R_a = V_a/i$; relating to changes in the electrolyte), and polarization impedance ($Z_p = V_p/i$; relating to changes at the electrode surface) [9, 24]. The iPSC colonies were manually cut into small samples, and then transferred into the custom cell culture and stimulation module. After one day of recovery, the iPSC stimulation began. The simulation of human iPSCs into neurons follows the same steps as shown in Figure 6-1A. At first, the iPSCs were stimulated every day up to four days and then once every two days until the tenth day. There was obvious morphological transformation from Day 4 to Day 10 as Figure 6-1B shows. The conventional colonies could be formed on the Matrigel-coated PPy-DBS film without stimulation compared with the stimulation groups, where there was neuronal-like elongation.
Figure 6-1  Schematic of human iPSC stimulation.

A) Schematic of cell culture and stimulation setup illustrating the major stages of the method for iPSC stimulation. Human iPSCs were seeded on the custom module for cell culture and stimulation with counter electrode and culture chamber (as the real module shown in Figure S1). B) Detailed time course of iPSC stimulation. The iPSCs started to be stimulated from Day 1 using alternating current at ±0.1 mA/cm² every day to Day 4 and then at ±0.25
mA/cm² every two days to Day 10. For each time, the cells were stimulated for 8 h per day period.

Figure 6-2 Electroactive PPy-DBS substrate.

A custom-made human iPSCs culture chamber and stimulation module comprising gold-coated Mylar film with polymerized PPy-DBS and platinum mesh counter electrode.

Figure 6-3 Cyclic voltammogram of PPy-DBS in PBS media with clear oxidation and reduction peaks.
6.3.2 The characterization of stimulated human iPSCs

The kinetic status of stimulated human iPSCs is illustrated in Figure 6-5A demonstrating a wide scope of morphological changes post ten days (Figure 6-5B). Immunophenotyping with confocal microscopy demonstrated neural differentiation of stimulated iPSCs in Figure 6-6, where pan neuronal-marker TUJ1 and VIMENTIN and glial cell marker GFAP were immunoreactive. The undifferentiated iPSCs, maintained the classical morphology with the expression of pluripotent stem cell markers OCT4, SOX2, and SSEA4 (Figure 6-7). RT-qPCR was used to corroborate immunophenotyping to quantify the effect of electrical stimulation on iPSC differentiation. This revealed upregulation of pan-neuronal and neuroglial markers. Specifically, electrical stimulation induced higher expression of SYNAPTOPHYSIN ($F (1, 4) = 3518.615$), TUJ1 ($F (1, 4) = 421.390$), GABA ($F (1, 4) = 940.309$), OLIG2 ($F (1, 4) = 93.018$) and SERT ($F (1, 4) = 1021.836$) with concomitant lower expression of OCT4 ($F (1, 4) = 1105.359$) and NANOG ($F (1, 4) = 1299.920$) (Figure 6-8). There was no difference between the astrocyte marker GFAP and GABAergic marker
GAD. Interestingly, SYNAPOPHYSIN expression was markedly increased. RT-qPCR also confirmed increased transcript for ectoderm germ layer marker *CERBERUS* (*F* (1, 4) = 831.632) and *HAND1* (*F* (1, 4) = 837.351), mesoderm marker *H19* (*F* (1, 4) = 7845.837) and *IGF2* (*F* (1, 4) = 414.442) with marked increase for *HAND1* (Figure 6-9).

![Figure 6-5](image)

**Figure 6-5** Time course of stimulated human iPSCs.

**A**) Images of stimulated human iPSCs on the PPy-DBS substrates at specific time points. **B**) Brightfield micrographs of human iPSCs on the PPy-DBS with stimulation (left) and with stimulation (right) 12 days post-first-stimulation.
Figure 6-6 Immunostaining of differentiated human iPSCs.

A) Immunocytochemistry showing GFAP and TUJ1 expression for stimulated human iPSCs with DAPI staining 12 days post-first-stimulation. B) VIMENTIN expression in the stimulated human iPSCs. Pseudocoloured images as indicated by colour text. Scale bars as indicated.
Figure 6-7 Immunophenotyping of human iPSCs on PPy-DBS substrates.

**A)** Cells stained with DAPI and expressed pluripotent nucleus marker OCT4. **B)** Cells stained with DAPI and expressed pluripotent nucleus marker SOX2. **C)** Cells stained with DAPI and expressed pluripotent membrane marker SSEA4. Scale bars as indicated.
Figure 6-8 Gene expression of stimulated human iPSCs on PPy-DBS substrates.

Comparative gene expression of neural markers between stimulated and non-stimulated iPSCs on PPy-DBS substrates 12 days post-first-stimulation. Relative gene expression represents data normalized to $\beta$-actin and expressed for the non-stimulated iPSCs. Mean ± S.D.; n = 3. One-way ANOVA with Bonferroni post hoc test. * $P<0.05$; ** $P<0.01$; ***$P<0.001$. 

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Endodermal and mesodermal gene expression stimulated human iPSCs on PPy-DBS substrates.

Comparative gene expression between stimulated and non-stimulated iPSCs on PPy-DBS substrates 12 days post-first-stimulation. Relative gene expression represents data normalized to \( \beta\)-actin and expressed for the non-stimulated iPSCs. Mean ± S.D.; \( n = 3 \). One-way ANOVA with Bonferroni post hoc test. * \( P < 0.05 \); ** \( P < 0.01 \); *** \( P < 0.001 \).

6.4 Conclusion

This work represents the first demonstration of stimulation for the improvement of iPSC differentiation without inducing factors such as BDNF and NGF. To the best of the PhD candidate’s knowledge, there is as yet no report of the stimulation of human pluripotent stem cells. Trials of electrical stimulation of human iPSCs have been carried out and found to have an effect on human iPSCs with successful generation of neuronal-like cells after approximately ten days of stimulation. The differentiation time frame has been shortened compared with conventional methods [25, 26]. Furthermore, the mechanism of stimulation
effects on the differentiation of stem cells needs further investigation. In summary, the results presented here have explored a new way to facilitate neural differentiation from human pluripotent stem cells.
6.5 References

Chapter 7

Conclusions and Recommendations

7 CONCLUSIONS AND RECOMMENDATIONS

7.1 Conclusion

The work presented in this thesis aimed to demonstrate the utilization of 3D printing with a novel bioink as well as conductive materials with electrical stimulation for the control of stem cell fate in order to generate functional tissues for future research and potential clinical application.

Direct-write printing of stem cells within biomaterials presents an opportunity to engineer tissue for in vitro modeling and regenerative medicine. Reported here is the first example of the construction of neural tissue by printing human NSCs that were differentiated in situ to functional neurons and supporting neuroglia. The supporting biomaterial incorporated a novel clinically relevant polysaccharide-based bioink comprising Al, CMC and Ag. The printed bioink was found to gel rapidly by stable crosslinking to form a porous 3D scaffold encapsulating stem cells for in situ expansion and differentiation. Differentiated neurons were spontaneously active, showed a bicuculline-induced increased calcium response, and predominantly expressed gamma-aminobutyric acid (GABA). The 3D tissues will facilitate investigation of human neural development, function and disease, and may be adaptable for engineering other 3D tissues from different stem cell types.

The ability to create 3D tissues from human iPSCs is poised to revolutionize stem cell research and regenerative medicine. There are, however, few examples of 3D tissue engineering using iPSCs, with in vitro stem cell culture and differentiation predominantly planar (2D) for either monolayer cell support or substrate-dependent induction of self-organizing embryoids and organoids. Although not previously described, bioprinting iPSCs with advanced functional biomaterials promises to augment efforts to develop discrete 3D tissues, ideally comprising direct-write printing of stem cells for encapsulation, proliferation, and successive directed differentiation. Such a method is described here. A clinically-
amenable polysaccharide-based bioink, Al-CMC-Ag, is the first example of bioprinting human iPSCs for \textit{in situ} expansion and sequential differentiation for 3D tissue fabrication. Specifically, this work has demonstrated extrusion printing of bioink encapsulating iPSCs for gelation to a stable and porous 3D construct, proliferation of the iPSCs within the construct and differentiation of the same iPSCs into either EBs comprising cells of three germ lineages – endoderm, ectoderm, and mesoderm, or more homogeneous neural tissues containing functional (including migrating) neurons and supporting neuroglia. It is envisaged that this fully defined, scalable and versatile platform will be useful in human iPSC research and translation for pharmaceutical development and regenerative medicine.

Finally, electrical stimulation has been applied towards organ repair in clinical settings in recent years. Stimulus-responsive biomaterials hold great promise for tissue engineering because an electrical signal is an important regulation method for embryo development and cell-cell communication. In this study, PPy films have been evaluated as substrates for human iPSC culture. Conventional colony morphology was observed. In addition, the films were used to stimulate human iPSCs. The results demonstrated a significant increase in neural differentiation compared with those that were not stimulated. These findings indicate the potential of stimulation for improving pluripotent stem cell differentiation.

### 7.2 Recommendations

Biomaterials contribute to stem cell support and derivative tissue architecture. The biocompatibility and nanoscale modification of materials will therefore benefit stem cell research and translation [1]. Currently, individual materials cannot satisfy all requirements of stem cells and so different monomers have been combined together with specific modification to be used in complex bio-research [2, 3]. Nonetheless, methods to direct stem cells into functional cells under 3D conditions are still under-explored. Composites of materials could assist with cell patterning of different cell types, which is essential for
fabricating complex tissues [4]. Furthermore, the mechanism by which each property of the materials influences stem cell regulation needs to be determined and encapsulation of the cells needs to be achieved without loss of viability or desired phenotype. Currently, the materials used for encapsulating stem cells are mainly hydrogels because hydrogels have the ability to hydrate and maintain the water content for cell survival. Intelligent materials may have additional properties under specific conditions (e.g., pH, electrical, pressure, magnetic and some others) to induce chemical signals, which have been found to induce the conversion of stem cell states [5]. An effective combination of the properties of biomaterials to stem cells into preferred cell types therefore represents the future of biomaterials interfacing with stem cells for 3D tissue engineering. An additional important requirement though will be incorporating vasculature to fabricate tissues with (micro) blood vessels Blood vessels in native tissue are essential to transport the nutrients and waste, but current materials and 3D prototyping technologies still cannot recapitulate \textit{in vivo} vasculature form and function. Nonetheless, potentially suitable biomaterials are being developed and studied towards the next generation of smart biomaterials towards having an entire gamut of suitable materials/properties to efficiently control stem cells in 3D and generate \textit{bona fide} human tissues and organs \textit{in vitro} for biomedical research and ultimately “smart regenerative medicine”.
Reference