NANYANG
TECHNOLOGICAL
UNIVERSITY

**3D PRINTING HYDROGEL SCAFFOLD AND
FUNCTIONAL HYDROGEL FOR TISSUE
ENGINEERING APPLICATION**

HU KAN

**SCHOOL OF MECHANICAL AND AEROSPACE
ENGINEERING**

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the degree of Doctor of Philosophy

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List of Abbreviations and Symbols

Abbreviations

3D	Three-dimensional
2D	Two-dimensional
ECM	Extracellular matrix
DOE	Diffractive optical element
PEGDA	Polyethylene (glycol) diacrylate
rGO	Reduced graphene oxide
LbL	Layer-by-layer
PEG	Polyethylene glycol
FU	Functional unit
DMD	Digital mirror device
IL	Interference lithography
HL	Holography lithography
HA	Hyaluronic acid
BG	Bioactive Glass
TCP	Tricalcium phosphate
CPC	Calcium phosphate
PGA	Polyglycolic acid
PLA	Polylactic acid
PCL	Polycaprolactone
FDA	US Food and Drug Administration
MMps	Matrix Metalloproteinase

I2959	Irgacure 2959
TEMPO	2,2,6,6,-Tetramethylpiperidine, 1-oxy
FESEM	Field emission scanning electron microscope
FTIR	Fourier transforms infrared spectroscopy
UV-Vis	Ultraviolet-visible
ASTM	Subtractive manufacturing methodologies
CAD	Computer aid design
FDM	Fused deposition method
EBM	Electron beam melting
DLP	Digital light processing
2pp	Two-photon polymerization
DMD-PP	Digital mirror device-based projection printing
FDM	Fused deposition modeling
CIJ	Continuous inkjet
FCC	Face centered cubic
EUV	Extreme ultraviolet
EBL	Electron Beam lithography
N.A	Numerical aperture
1D	One-dimensional
FCT	Face centered tetragonal
PNIPAm	Poly (N-isopropylacrylamide)
HEMA	2-hydroxyethyl
HPMA	2-hydroxypropyl methacrylate
AAM	Acrylamide
ESP	Enzyme-sensitive

GFs	Growth factors
KDP	Kys donor peptide
BMP	Morphogenetic protein
PLGA	Poly (lacti-co-glycolic acid)
HPMC	Hydroxypropyl methylcellulose
ROS	Reactive oxygen species
ERK	Extracellular signal regulated kinase
TEP	Trans-epithelial potential
TGF- β	Growth factor β
ALP	Alkaline phosphatase
BMPs	Bone morphogenetic proteins
PPy	Polypyrrole
GelMA	Gelation methacrylate
CSMA	Methacrylated chondroitin sulfate
MPEG-PCL-AC	Methyl ether-caprolactone-acryloylch
PEDOT	Poly (ethylenedioxy thiophene)
GO	Graphene oxide
DMF	N,N-dimethylformamide
PVA	Poly (vinyl alcohol)
HDMS	Hexamethyldisilazane

Symbols

E	Electromagnetic wave
E_i	Wave amplitude

k_i	Wave vector
φ	Phase of the beam
J_A	Molar flux of the drug
D	Diffusion coefficient of the drug in the hydrogel
C_A	Drug concentration
K	Ratio of drug concentration in the gel
δ	Hydrogel matrix thickness
k_1	Constant numbers
k_2	Constant numbers
m	Constant numbers
M_t	Amounts of drug released at time t and
M_r	Drug released at equilibrium.
Mz	Focal length of the lens,
M	Number of the phase levels.
h	Step height
f	Incident light frequency
Δ	Ratio of the period of the binary Fresnel lens
λ	Incident light wavelength
r	Distance from the optical axis
L	Overall size of the lens
f	Minimal focal length
n	Refractive index of the lens
n_0	Refractive index of the hydrogel
(r_m, z_m)	Coordinate of the internal points of the annulus
w	Width of the focused beam

d_{min}	Minimum feature size of the binary Fresnel
I_{peak}	Peak intensity of the focused light
$I_{incident}$	Intensity of the incident light
α	Constant number
γ	Constant number
E_1	Plate modulus of the hydrogel
t_1	Thickness of the crosslinked hydrogel
η	Ratio of the thickness of the polymer to that of the cover slip and
α	Ratio of the plate modulus of the polymer to the cover slip.
I_{pk1}	Peak intensity at top layer
I_{pk2}	Peak intensity at bottom layer
α	Inhibition rate
Abs_{max1}	Maximum absorbance of the light at top layer
Abs_{max2}	Maximum absorbance of the light at the bottom layer
$R_{polymerization}$	Polymerization rate
V	Voltage across the inner probe,
I	Current applied to the outer two probe,
w	Height of the thin film
s	Space between each probe.
$G_7(\frac{w}{s})$	Correction divisor function

Abstract

Ever since its development, tissue engineering has played a very significant role in the medical arena with an ever-growing demand for various tissue donations. One crucial factor in conducting *in vitro* tissue engineering study is the construction of a desirable artificial three-dimensional (3D) tissue scaffold to act as the extracellular matrix, meeting the complex requirements for specific cell cultures. In view of this, to achieve a more optimal scaffold for tissue engineering, this study addresses two major challenges in tissue engineering nowadays: scaffold fabrication techniques and developing functional hydrogel material for tissue scaffold fabrication.

Existing hydrogel scaffold fabrication techniques and systems utilized in constructing extracellular matrix (ECM) are either two-dimensionally limiting or expensive and time consuming. For instance, some simple fabrication methods fabricate structures with morphologies that cannot be accurately controlled, while others may introduce harmful organic solvents into scaffolds during the fabrication processes. In this study, a new 3D printing system, is presented which is capable of printing hydrogel scaffold structure with improved efficiency. The outstanding features of this system are mainly attributed by the transparent diffractive optical elements (DOEs) of linear binary Fresnel lens fabricated to control the luminous intensity distribution. These DOEs of different patterns are arranged in series on a cover slip with each optical element designed to focus and diffract light at a particular plane within the device. Coupled with other components of the system, 3D hydrogel cellular scaffolds can be easily printed via effective and efficient one-step light exposure to the photocrosslinkable polymer solution upon demand. The combined outstanding features of photocrosslinking and diffractive optical technique incorporated within this system enable the patterning of hydrogel within seconds for not only bulk structures, making large-scale fast production feasible, but also for localized, specific areas of interest, making printing of complex features simple. With this

system, 3D two-layered hydrogel woodpile structures were successfully fabricated within 3 seconds in this research.

Though the development of conventional *in vitro* 3D structural support properties of scaffold is crucial, the interests in recent years has steered towards developing multifunctional scaffold to achieve similar biochemical and biophysical features of the natural ECM for further regenerative medicine use. Among various functionalities explored in scaffolds so far, electric stimuli-responsive hydrogel integrated with controllable drug delivery technique has been a state of the art technology for scaffold fabrication. Electric signal plays significant roles in the cell differentiation and signaling among multiple tissues such as nerve, bone and cardiac etc. More importantly, the regeneration process of these tissues needs the cooperation of multiple growth factors, drugs or biomolecules to be delivered to the localized area. Being a pilot study, another focus of this project was placed on investigating ‘smart’ conductive biomaterial that is capable of transiting electric signal as well as delivering incorporated drugs. We integrated hydrogel polyethylene (glycol) diacrylate (PEDGA) with water-soluble reduced graphene oxide (rGO) and created a new trend of ‘smart’ photo-patternable conductive hydrogel. This photo-patternable conductive hydrogel is capable to be printed as conductive 3D tissue scaffold, serving as a conductive 3D template to provide external supports and electric stimulations for cell growth, what is more, its therapeutic controllable drug-delivery capability also allows the hydrogel to be loaded with varied biomolecules, becoming a therapeutic agents carrier, releasing them when needed in a controllable manner. The release of the incorporated agents can be easily achieved via the application of external electric signal with ‘click’ action. Layer-by-layer (LbL) poly-arginine *siRNA* nano-particles were fabricated and incorporated into the hydrogel, followed by electric stimuli assisted release test. The released nano-particles were then treated to mouse fibroblast cells to confirm if the *siRNA* loaded nano-particles still retain bioactivity. Confocal microscopy showed successful uptake of the nano-particles into the cells. Western blotting of the treated cells lysates showed intended protein knockdown, indicating that the released nano-particles retained their

functionalities, proving the conductive hydrogel's capability to safe release of sensitive agents. Therefore, this composite polymer is both a favorable cell culture construct and a drug delivery vehicle.

Chapter 1 Introduction

1.1 Background

1.1.1 Tissue Engineering

Since ancient times, man has always dreamt of attaining ever-lasting life through the rejuvenation of damaged human body parts. Man has strived to develop the art of engineering tissue throughout the centuries. The first recorded case of human tissue replacement occurred in the 16th century [1]. Tagliacozzi of bologna, Italy, conducted a nose transplant. With the development of life science and surgery in the 19th century, Man gained more understanding in the human anatomy and practical experience in the surgical field, the advanced whole human therapy transplant seems to be more realistic [1, 2].

In the recent years, tissue engineering has developed to become an area of paramount importance in the medical and biology field. Every year there are millions of people with various tissue diseases waiting for organ transplants or treatments [3]. Some areas of treatment includes the skin, cartilage, bone, liver, blood vessels, heart, etc. [1]. With a large demand for tissue and organ replacements, lacking of organ supplies to meet the high demands makes it difficult for patients to receive proper and timely treatments. Even for patients who receive organ donations, there exist high operation failure risks due to rejection from the donated organ transplants [3]. Furthermore, due to the scarcity in organs, various social and ethical issues are emerging. The security of some organ sources is compromised due to the difficulties in controlling the organ harvesting processes and the verification of the sources and qualities [1]. These problems can be easily solved if tissue engineering is reliable and readily available.

Tissue engineering is a combined application of the principles and methods of engineering and life science [3]. It is a product from the development and fusion of material science, engineering and life science [1, 3, 4]. The aim of tissue engineering is to produce a bio-product that can maintain, fix or even replace the functions of some damaged organs [1, 3, 4]. Tissue engineering consists of three processes: cells with specific functions separated from the human body will be cultured *in vitro* to achieve desired cell proliferation and differentiation. Subsequently, these cells will be seeded on a scaffold for further development to construct the biological substitutes [3]. Finally, the scaffold can be transplanted into the targeted lesion site of the tissue in question to maintain, improve or repair tissue functions [1].

1.1.2 Tissue Scaffold

One significant factor that makes tissue engineering possible is the reconstruction of suitable *in vitro* environment matrixes that serve as scaffolds for the attachment of cells to proliferate and migrate [1, 3, 5, 6]. To illustrate the importance of suitable scaffolds, the analogy in the case of construction works to build an erection can be made. To build an erection, the basic materials required are cubic stones and metal frames. The metallic spokes inside a building serves as the rigid frame used in supporting the whole building. Millions of stacked stones are then cemented and held together by these metal frames to form the final outlook of the building [3]. As a comparison in the field of tissue engineering, for a functional organ or tissue, the cells can be seen as the cubic stone while the metal frames can be compared to the scaffold which supports the whole organ or tissue. Different organs possess varied shapes and functions. Thus, one of the main challenges of tissue engineering will definitely be the fabrication of a suitable supporting ‘metal frame’ with the desired specific three dimensional shape and pore size unique to the organ in question, which allows not only the free migration of the cells through the interconnections but also adequate access of minerals and nutrients

to the cells for optimized growth [3]. Furthermore, the materials used for the scaffold should possess specific mechanical and chemical properties to meet the demand of cell attachment, proliferation and growth. For instance, the natural tissue stiffness for liver is around 2 KPa; while for cortical bone tissues, the stiffness requirement is 10 GPa [2, 3, 5, 7-9].

There are many technologies and systems developed by researchers to fabricate suitable tissue scaffold. Solvent casting, phase separation and fiber bonding are some examples of fabrication methods [5, 6, 10]. These methods enable fast porous structure production enabling scaffolds to be fabricated within few hours [5, 6, 10]. 3D printing, another representative fabrication method, could print very defined structure with resolution down to micrometers [2, 6]. These defined structures could provide stable external environment for the studies of cell migration, alignment and proliferation [2, 6, 11, 12]. Based on these technologies, varied materials are utilized for scaffold fabrication. Researchers found that natural polymeric materials possess great potential in tissue engineering for their excellent biocompatibility [3]. However, limited material sources and poor mechanical properties are some of their drawbacks [13]. To overcome the limitations of natural materials, artificial polymeric materials are developed. Artificial polymeric materials have better mechanical properties as compared to natural polymers [14, 15]. However, the biocompatibility of the artificial material is poor comparison to natural polymers [15]. In this research, several advantages of existing technologies are combined to build up an ultra-fast 3D scaffold printing system. The systems are easy to set up and the printing processes are ultra-fast (few seconds). The fabrication materials utilized in this research are from hydrogel polyethylene glycol (PEG) family. The material integrates the advantages of both natural polymeric materials and artificial polymeric materials. Their mechanical properties can be varied. For instance, their strength can be controlled between 100 Pa to 100 KPa [15-17]. Also, as they are hydrogels (water based polymer networks), their biocompatibility properties are excellent [3].

1.2 Problem Statement

1.2.1 Tissue Scaffold Fabrication Limitation

Specifically, four major drawbacks of existing techniques for scaffold fabrication are noted and tackled in this work. Firstly, the fabrication techniques widely used previously are two-dimensional and serial in patterning [5, 8, 18, 19]. For example, microfluidic creation of stiffness and photo polymerization (using transparent masks) techniques enable only 2D cell behavior studies [5, 6]. However, functional units (FU) are the basic elements that form the entire tissue and these functional units normally possess different 3D architecture morphologies [20]. Thus, it is crucial to build up similar artificial microstructures, which is in a 3D manner to mimic the *in vivo* tissue micro-architecture and cellular microenvironment. This is the basic requirement for tissue engineering. Therefore, it is evident and crucial that the first target of the project is to create a 3D scaffold structure.

Secondly, most of the current techniques cannot precisely control the resolution or the shape of the scaffolds during fabrication [3, 4, 8, 11, 12, 21, 22]. 3D scaffold fabrication attempts developed by researchers (for example by gas foaming and fiber bonding) today usually produce scaffolds randomly interconnected together. For instance, for electro-spinning method, melted polymer is randomly ejected from the nozzle, and after solidification and drying, these polymer forms nano-fibers [23-25]. These ejected nano-fibers are usually connected with no order. Thus, it is very difficult to control the pore shape, not to mention the pore size or the resolution of the pores [26]. In the field of cell biology, cell migration strongly affects pathological phenomenon and tissue regeneration such as wound healing and immune responses, these activities all rely on the migration of specific cells from one location to another specific location. Therefore, failure of such migration may lead to serious consequences [27]. Various studies have shown that cell immigration is governed by cellular

interconnections, scaffold pore size and shape [28-31]. For example, mesenchymal stem cell attachment ability is directly related to the scaffold mean pore size [28], chondrogenic gene expression and cartilage matrix deposition depends on the pore size and shape [30]. Also, researchers observed when the scaffold pore size increases from 90 to 150 μm , the fibroblast migration speed decreases dramatically [28, 30, 31]. Due to above reasons, applications for these scaffolds are usually very limited, and it calls for a new fabrication system that could manually control the resolution of the pores and the shape of the pores. This will be investigated in the project.

Thirdly, many available 3D fabrication technologies that can precisely control both resolution and shape of the structures fabricated usually employ systems that are very expensive with extensively time-consuming manufacturing processes [32]. For instance, the stereo-lithography system (either for direct-laser manufacturing or mask based indirect laser manufacturing) usually utilizes scanning nozzles or laser beams which are very expensive components [2]. On the other hand, a normal 3D printer nowadays with regular printing resolution can cost up to million dollars [33, 34]. Furthermore, the commercial 3D printer's patterning ability has been limited to specific regions due to the size of the 3D printer (such as Zortrax M200, Poland, maximum printable structure size is 200×200×180 mm), therefore rendering large area fabrications impossible economically [2, 12, 17, 35]. Researchers have integrated some digital mirror devices (DMD) with the stereo-lithography system [35], trying to control the stage movement and therefore improve the productivity. However, the system precision is reduced as well [34]. Interference lithography is a newly developed method to fabricate the 3D microstructures. Two approaches to interference lithography are noted, multi-beam interference lithography (IL) and mask based interference lithography (holographic lithography) or phase mask lithography [36]. For multi-beam interference lithography, multiple coherent laser beams are directed to spatial point for interaction. Interference pattern are created due to the wave phase and intensity difference. In the field of lithography, these patterns can be recorded onto photo-resist (such as hydrogel), preserving enhanced intensities while eliminating the weakened intensity. Thus, the

intensity variation appears as periodic pattern on the photo-resist. The number of the light beams determines the final interference pattern and more light beams create more complex 3D periodicity [36, 37]. Holographic lithography (HL) utilizes the hologram to record and preserve original structures; original 3D image could be reproduced once upon one-step reconstruction light exposure. Overall, fabricating time for IL/HL is usually shorter as compared to 3D printing, and it can control the feature size in the lateral and vertical directions accurately [36, 37]. So far, the IL/HL technique is mostly used in the field of semiconductor and optical system. Not many researches have explored the possibility of IL/HL technique in the fabrication of tissue scaffold [38]. Of the few researchers that explored this area are Lasagni et al. [38] who introduced femtosecond pulsed laser to fabricate 2D structures with PEGDA using IL technology. They managed to produce 2D pillar and mesh structures. However, their systems are fairly expensive due to their choice in light sources. Another group is Kang's group [39] which utilized a prism to divide one laser beam into several beams to create interference pattern for photo-crosslinking poly (HEMA-*co*-MMA) hydrogel 3D scaffold fabrication. However, HL stands to be a better fabrication choice than IL. This is because as compared to IL, HL technology does not rely on multiple laser beams to produce interference patterns. Therefore, accurate system positioning is not strictly required, largely reducing system costs and promoting improved stability extensively [38, 40-42]. Also, by integrating customized hologram, the recorded structure complexity is not limited to periodic.

This research discusses the possibility of borrowing the idea of holographic lithography to build a printing system for fast, complex 3D structure printing within PEGDA hydrogel. By utilizing HL technology, we aim to print 3D structure in the hydrogel with one-step exposure. A printing system was built enabling cheap and one-step exposure, fast printing of 3D woodpile hydrogel structure. Transparent linear binary Fresnel lenses (DOE) with different focal points were designed and fabricated for patterning woodpile hydrogel structure. As compared to traditional phase masks (phase gratings), which can only produce certain periodicities, binary Fresnel lenses can focus light

onto any designed plane, largely improving the capability of producing structures of varying sizes [43]. Another advantage is the ease of the manufacturing of the binary Fresnel lens, which can be simply achieved by photolithography. Two-layered woodpile structures were printed utilizing two different linear binary Fresnel lenses for the first time. The morphology of the structures was then tested with phase contrast microscope and field emission scanning electron microscope. On top of that, printing time and chemical concentration was modified to investigate the effects of these parameters on the quality of the printed products. Finally, cell toxicity test was conducted to confirm the biocompatibility of the printed scaffolds.

1.2.2 The Emergence of Functional Electric-Stimuli Responsive ‘Smart’ Biomaterial for Tissue Scaffold

Meanwhile, the paradigm of tissue scaffold design and fabrication nowadays has no longer been solely emphasized on developing biocompatible *in vitro* structural support by simply manipulating the scaffold porosity, surface morphology and the shape [44], but more towards developing multifunctional scaffold to achieve the imitation of both biochemical and biophysical of the natural ECM for further regenerative medicine use [45-49].

The human body is governed by various biophysical and biochemical stimuli (chemical, mechanical, material and magnetic etc.) to maintain the structural and functional stability [50]. Nowadays researchers are exploring the possibility to apply these stimuli to *in vitro* models to further control and improve tissue implants [46, 51]. Among all the factors, electric stimulation assisted hydrogel scaffold fabrication and drug delivery have been active topics in recent research. Varied studies have proved electric field not only has strong effects on nerve and muscle functions but also plays a significant role in the maintenance of cellular functions [52], tissue scaffold morphologies [52-55], cell proliferation, cell migration, gene expression and wound healing [47, 54-56]. Thus, due to the

broad influence of the bioelectricity and its extraordinary effect on tissue regeneration, electric responsive hydrogel scaffold has been created for tissue engineering applications [57]. Specifically, studies show that electrical pulse functions as a stimulator to the regeneration process of nerve cells, as the current passing through the nerve cells alters the polarization of the cell membranes [47]. Also, researchers have proved that increasing electric stimulation leads to a positive effect on the muscle cell growth rate and differentiation rate [55]. On top of that, osteo-conductivity and angiogenesis have been the main concerns for bone tissue scaffold design [58], as bone tissues require the participation of growth factor at the local injury sites to initiate and promote the healing process [58]. What is more, *in vitro* cardiac tissue engineering has been a challenging research topic due to the strict electro-mechanical property requirement for the tissue matrix. The synchronized movement of the cardiomyocytes requires complex electrical connectivity among each individual cardiomyocytes [54]. Besides achieving electrical connectivity, the regeneration process also needs the cooperated function of multiple growth factors, drugs or biomolecules to be delivered to the localized area, achieving high local dosages while preventing the destruction of the agents during the delivery process [58, 59].

Evidently, conventional simple hydrogel scaffold without further characterization cannot meet nowadays tissue scaffold requirements. Therefore, functional hydrogel and controlled drug release technologies are being introduced to the field of tissue engineering [51, 58, 60]. Developing multifunctional hydrogel scaffolds which can not only provide stable structural and physio-chemical environment for cellular support, but also act as ‘smart’ therapeutic agent-release regulators to control the dose and kinetics of released agents at the local injury site is a highly desired goal for tissue engineering [58, 61, 62].

In this research, we have developed a state of the art photo-patternable “smart” conductive hydrogel. This new generation of composite polymer not only keeps the varied advantages of the conventional hydrogels (such as hydrated nature, photo-patterning ability, tunable tissue-like surface

stiffness, and excellent biocompatibility [59, 63, 64]), but also makes direct delivery of electric stimulations to implanted cells realistic. Furthermore, the easy to tailor property of the hydrogels enables them the capability to meet the specific needs of designed applications simply via adding of biomolecules, enzymes, antibodies, nano-particles, growth factors or drugs. Thus, this photo-patternable conductive hydrogel offers the promise to be fabricated as conductive 3D tissue scaffold, serving as a 3D template to provide external support for the cells. At the same time, it enables the direct application of electrical stimulation to cells. Furthermore, its drug-delivery capability also allows the hydrogel to be loaded with drugs or biomolecules, becoming a therapeutic drug or biomolecules carrier, releasing the agents accurately and on-demand doses of medication at a specific tissue site when needed in a controllable manner. The controllable release kinetic can be easily achieved via the aid of external electric stimuli with simple “click” action.

1.3 Research Objectives and Organization

Based on the problem statement mentioned above. The objectives of this study are to address the two major challenges in tissue engineering study nowadays: scaffold fabrication techniques and developing functional hydrogel material for regenerative tissue scaffold application.

1.3.1 Originality

The thesis involves the research and development in the following four aspects:

1. A new 3D printing system is designed and established. The system is capable of printing 3D hydrogel scaffold with improved efficiency (less than 3 seconds).
2. The printing ability and the limitation of the system are tested.

3. A new conductive hydrogel biomaterial is developed. The biomaterial is able to transit electric signal and release the incorporated substance under electric field control.
4. The effect of electric field on the material surface morphology and drug release ability are systematically studied.

1.3.2 Significance and Scope of the Research

The major challenge in hydrogel tissue scaffold fabrication technique is that the current popular fabrication technology/system either cannot precisely control the scaffold resolution/integrity or is very time consuming and expensive [5, 7, 8, 12, 26]. For instance, stereo lithography usually utilizes scanning nozzle and builds up the structure with long layer-by-layer scanning process [65]. On the other hand, two-photon polymerization, another advance technology being utilized for scaffold fabrication, although could produce structure feature size down to nanometer, the complicated optical system and expensive laser sources render its practical use [66]. Therefore, these limitations all call for a new system to be developed for tissue scaffold construction. The new system should be able to control the integrity, complexity and resolution of the structure in a low-cost, fast, large area fabrication manner.

Mask-based IL technology harnesses the advantages of high-precision, fast fabrication with affordable optical system, DOE design and integrates them together to offer greater flexibility for 3D structure fabrication. We aim to build an ultra-fast 3D printing system with the aid of masked-based IL technology. The system will be able to produce the whole hydrogel structure upon one-step reconstruction light exposure of the DOE, in a highly improved efficiency (less than 5 seconds) down to micrometer resolution. When compared with other hydrogel scaffolding system, this ultra-fast printing system proves to be efficient and cost-friendly. Following strategies would be approached in order to achieve the objectives of the first project:

1. Photo-patternable PEGDA hydrogel would be selected as the fabrication material for the printing system.
2. Photolithography would be utilized to produce the linear planar step-like microstructure on wafer (to approximate a spherical phase front).
3. The DOE pattern on wafer would be transferred to cover slip by nano-imprinting, serving as the DOE. The designed DOE would be light and can be easily replicated from the wafer mould.
4. Further, we would integrate the DOE with other optical components and establish the new printing system. The system would be able to print scaffold automatically with customized program.
5. Printed hydrogel scaffold would be characterized for their surface nano-mechanical property, structure bonding strength and cell biocompatibility.
6. Evaluation of factors affecting the printed scaffold's resolution would be conducted.

What is more, another major challenge in hydrogel scaffold fabrication is that the traditional hydrogel materials (such as PEGDA) can no longer meet the demands for nowadays' regenerative tissue engineering requirements. Various cell types and tissues ask for different conditions (such as electrical signaling, growth factor/therapeutic drug delivery) for optimal cell growth and differentiation [67]. For instance, bone tissues require the participation of growth factor at the local injury sites to initiate and promote the healing process [68]. The synchronized movement of the cardiomyocytes requires complex electrical connectivity among each individual cardiomyocytes [69]. Therefore, the application of the 3D printing system established in the first project is still largely limited by the usage of conventional PEGDA hydrogel. This is due to PEGDA hydrogel lacking the bio-conductivity and drug delivery ability. Therefore, in the second project, biocompatible, high concentration water-soluble rGO would be combined with PEGDA to create a state of the art conductive hydrogel scaffold material. The combination of the two materials harnesses the advantages of both materials, integrating the advantages together to offer greater flexibilities in fabricating a new type of bio-responsive scaffold. When compared with traditional hydrogel material, this biocompatible

composite rGO/PEGDA system is proved to be able to transit electric signal and deliver sensitive incorporated drug/particles under electric field control. This new biomaterial reinforces its promise for regenerative tissue engineering research. Following strategies would be approached in order to achieve the objective of the second project.

1. Specific modification of rGO would be made with L-Lysine to increase its solubility to hydrogel. The modified rGO would be integrated with PEGDA and crosslinked via UV light.
2. Field emission scanning electron microscope (FESEM), Fourier transform infrared spectroscopy (FTIR), Raman spectroscopy, ultraviolet-visible (UV-Vis), four probe testing and cell toxicity test would be utilized to characterize the composite hydrogel.
3. A release system would be designed for the electrical field assisted drug delivery model. Where the electrical field played the role of a 'porogen' to the composite hydrogel scaffold. It mediated the permeability of the hydrogel, modified hydrogel surface structure, created porous and induced the diffusion and release of the particles.
4. LbL poly-arginine FITIC tagged *siRNA* nanoparticle would be fabricated and incorporated into the hydrogel to conduct particle release study under electric field. The released amount of nano-particle would be evaluated with Tecan machine.
5. Evaluation of electrical parameters affecting the composite hydrogel surface morphology would be systematically conducted with FESEM.
6. The released nanoparticles would be further examined with confocal microscopy and western blot to confirm the protein knock down effect.

Overall, this dissertation consists of the following 5 chapters.

Chapter 1 presents a brief introduction on current progress of tissue engineering studies; in particular, tissue scaffold fabrication technique and functional hydrogel are specifically discussed. It highlights

the motivations of conducting this research after reviewing the limitations and problems of current researches. The objective and the outline of the entire dissertation is presented as follows:

Chapter 2 presents a detailed review of the current research in hydrogel tissue scaffold fabrication and emergence for functional hydrogel material development. The advantages and disadvantages of these fabrication technologies will be elaborated upon, among which, 3D printing and interference lithography are extensively discussed. Then, the role of bioelectricity phenomenon in the human body was detailed explained and the emergence of developing functional printable electric-stimuli responsive hydrogel for tissue engineering applications was specifically discussed.

Chapter 3 reports the establishment of a 3D hydrogel scaffold printing system for fast, one-step printing of hydrogel scaffold. Two-layer hydrogel scaffold was printed with this system in less than 3 seconds. Optimization of crosslinking condition of the printing process was carried out to achieve more defined hydrogel structures, and the results and discussion are stated.

Chapter 4 establishes a state of the art functional electric-stimuli responsive hydrogel for tissue scaffold applications. This ‘smart’ photo-patternable functional hydrogel owns the ability to release nano-particles upon electric field control. Electric field induced nano-particle release study was conducted. *In vitro* cell test of the released nano-particles was systematically studied to prove the conductive hydrogel’s capability to safe release of sensitive agents.

Chapter 5 draws the conclusions from the above findings and suggests future work for next stage.

Chapter 2 Literature Review

2.1 Requirements of Scaffold Fabrication

To fabricate a desirable scaffold, various requirements should be fulfilled. Basically, five requirements for the fabrication of tissue scaffold are noted [1, 3] and they are:

1. Complex biological safety requirements. As living cells are to be cultured in these tissue scaffolds, the scaffolds should be non-toxic, providing a suitable environment for cell growth.
2. Suitable biodegradable and absorbable ability. Once the scaffolds seeded with cells are planted into the damaged organs in the human body, tissue growth within the scaffolds will replace or repair the damaged parts of the organs. After the healing process, the scaffolds should be able to degrade so the patients will not have to undergo additional surgeries to remove the scaffolds upon recovery. Thus, the biodegradable and the absorbable properties of the scaffolds are of vital importance during the fabrication of tissue scaffolds [3, 5, 70].
3. Suitable pore size, high porosity and connected pores [5, 7, 71, 72]. According to cell biology, cells need to communicate with each other to confirm the environment they are in is suitable for their survival [27, 28]. For instance, proteases are only effective on the surfaces of the scaffold; thus, the surface area and porosity of the scaffold have crucial effect on the degradation rate of the scaffold [3, 15, 73, 74]. Besides, the shape of the pores controls the growth rate of the tissue; desired pore size could enhance cell activity. However, the choice of the shape and size of the pores also depends on the type of cell cultured in the scaffold [13, 72, 73]. For instance, suitable pore size for bone tissue engineering would be around 75-250 μm , while for the fibrocartilage tissue, the recommended pore

size range is 200-300 μm [70, 72]. Therefore, an optimal pore size in the scaffold will enable cell movement and information transformation, promoting cell proliferation [30].

4. Specific 3D shape with structure integrity [3, 5, 73, 75]. As different organs have different shapes, the shape of the scaffold should be customized accordingly to simulate the appropriate cellular environment.

5. Big surface area and suitable physical and chemical properties [2, 7, 13, 72, 76]. The scaffold used to replace a damaged area within an organ should provide similar physical, chemical and mechanical properties as the intended target area within the organ. Cellular behaviors depend strongly on their surface roughness and mechanical environment. For instance, the stiffness of the environment helps determine how stem cells differentiate while the gradients of stiffness trigger migration (durotaxis) while rough surfaces can improve the speed of cell proliferation, speed up the facilitation of vascular vessels, improve the supply of oxygen and nutrients and help eliminate waste. Suitable malleability and mechanical strength are also required to maintain the shape of the tissue for a certain period of time after the implantation for new tissue formation [3, 11, 15, 72-74, 77]. It is therefore important to be able to control and pattern the stiffness of the ECM as well as its permeability and chemical compositions [3, 7, 13, 71, 75, 78-80]. One woodpile hydrogel scaffold is shown in figure 2-1.

Figure 2-1 A two-layer PLA (polyactide) woodpile scaffold cultured with PC12 neuroblastoma cells [76].

The priority function of tissue scaffold is to serve as the ECM. ECM is mainly composed of collagen (insoluble filamentous fibres), water and proteoglycan. Therefore, the tissue scaffold usually utilizes polymeric materials to simulate some of the important properties of the ECM. Overall, the materials used for tissue scaffold fabrication can be categorized into two groups according to their sources namely: natural polymeric materials and artificial materials [5, 15, 74, 76, 79, 81, 82].

2.2.1 Natural Polymeric Materials

Natural polymeric materials generally have high organic combinations and also contain ligands, facilitating the integration of cells and receptor proteins [71, 79, 80]. Although these materials have good biocompatibility, in some case, they may also cause inflammation. Natural polymeric materials mainly refer to collagen, coral, alginate, chitin, chondroitin sulphate, fibrin, bone matrix gelation and animal bones [3, 19, 80, 83]. Previously, researches focused on using decellularized technology to produce natural ECM. Natural ECM usually contains specific cell surface receptors for recognition, which could help to eliminate the immune rejection. Also, natural ECM could be bound with complex growth factors, promoting cell growth and differentiation [83]. Furthermore, comparing to artificial tissue scaffold fabrication, fabricating natural ECM does not need to consider the control of pore size and pore diameter [84]. However, the source of these natural ECM is limited, which makes wide application economically unrealistic. Meanwhile, utilizing natural ECM may also cause certain risks like the spreading of infectious diseases. Furthermore, some natural ECMs does not have strong mechanical strength to support cell attachment while others suffer from rapid degradation. Thus, using natural ECM for tissue engineering is still very challenging [6, 84].

Collagen is a kind of fibrous protein, which is a main component that connects tissues. It has been widely used in tissue regeneration, especially in soft tissue repair. Its cell recognition function can regulate cell attachment. However, it can also potentially cause adverse immune reactions [1]. In

order to reduce immune response, collagen must undergo purification treatment before cell implantation. However, even with the purification process, there are still high risks that bacteria may be introduced, causing side reactions. On the other hand, collagen has low mechanical properties, making the scaffold fabrication process difficult to be controlled [3, 5, 9, 85].

Alginate is a polysaccharide extracted from algae. It could be manufactured into gel or granular forms for cartilage repair. Utilizing alginate to wrap chondrocytes can maintain the functions of the chondrocytes and enable the dedifferentiation of monolayer cell cultures [1]. Liquid alginate is injected into calcium to initiate the crosslinking process, and at the same time, inhibiting the migration of defect and maintaining structural morphology. However, the fast degradation rate of alginate and its inadequate mechanical strength deem the structures made from alginate unsuitable for long-term implantation use [3, 68, 78].

Agarose is another polysaccharide extracted from algae. It can be processed as hydrogel. Cells are able to grow evenly and well distributed on scaffolds made of such hydrogels. Agarose can also be fabricated as injectable scaffold for cartilage, providing desired 3D mechanical environment for the cartilage. However, similar to alginate, its degradation rate is very fast. Agarose has been widely applied to the *in vitro* behavior studies of chondrocytes. It is reported that upon compression, agarose could transfer the mechanical forces to chondrocytes, stimulating the cells to secrete more extracellular matrix [1].

Fibrin colloid is obtained by curing a mixture of fibrinogen and thrombin. Fibrin colloid scaffolds can be fabricated using a patient's own fibrinogen and thrombin. Such scaffolds are biocompatible and the side effects caused by introducing these scaffolds into the human body can be neglected. Fibrin colloid is usually used to guide cell migration or crosslinked with other scaffold materials [1, 74, 86].

Hyaluronic acid (HA) is an anionic polysaccharide, which can be obtained directly from nature or microbial fermentation. Similar to collagen, HA can be chemically modified easily. Due to its non-immunogenic property, HA will not cause inflammation and body rejection. Therefore, it is a suitable biocompatible material for bio applications. However, its poor mechanical properties limit its application in scaffold fabrication [15].

Overall, natural polymeric materials have shown some advantages in tissue scaffold fabrication, however their drawbacks, such as poor mechanical property, fast degradation rate also largely limited their applications. In order to tackle all these problems, researchers have developed artificial polymeric materials.

2.2.2 Artificial Polymeric Materials

Comparing to natural polymeric materials, artificial polymeric materials possess better flexibility. They can be processed into different sizes and shapes. Their physical and chemical properties can be tuned easily. Their mechanical properties and degradation rate can also be adjusted by altering their synthesis methods [7, 15, 18, 19, 73, 78, 87-89]. The degradation of these polymeric materials is generally accomplished by simple hydrolysis, making scaffolds made from them to have controllable degradation rates [73].

Artificial polymeric materials can be further categorized into inorganic biomaterials and organic polymeric materials [73, 90]. Synthetic inorganic biomaterials mainly refers to bioactive ceramics [91]. It is considered as one of the best alternative materials to bone tissue due to its excellent biocompatibility and high mechanical strength. Bioactive ceramics mainly include bioactive glass (BG), hydroxyapatite (HA) and tricalcium phosphate (TCP). Recently, researchers also developed a new self-curing material, calcium phosphate (CPC), which has good biocompatibility, easy application ability and stable performance characteristics. More importantly, it can self-harden in

human body, crystallizing to HA [15]. However, calcium phosphate ceramics also have wide limitations, such as poor mechanical properties, low flexural strength and low load bearing ability [15].

The most widely investigated organic polymeric materials include polyglycolic acid (PGA), polylactic acid (PLA), PGA/PLA mixtures, polycaprolactone (PCL) and polyethylene glycol (PEG) [1, 13-15]. PLA is a kind of polyester that has been applied in the medical field for years as human implants. It has received US Food and Drug Administration (FDA) certification for safe usage [72]. Due to its drainage property, PLA surface does not absorb water easily, reducing its rate of hydrolysis. Therefore, the degradation rate of PLA is relatively low, ranging from 12 months to 2years.

PGA is the simplest linear aliphatic polyester. It has high degree of crystal structure [92], high melting point and low solubility in organic solvents. The degradation rate of the PGA is very fast, making its processing difficult. However, the human body can easily absorb PGA due to its fast degradation rate. PGA has relatively higher tensile strength and modulus of elasticity compared to PLA. For scaffold fabrication, it is usually pulled into wires and form mesh discs structures [13]. Overall, due to their poor mechanical properties and fast degradation rate, PLA and PGA scaffolds are unable to mimic similar EMC environment of hard tissues.

PLA-PGA copolymer can vary the rate of hydration and hydrolysis by changing the ratio of its composition [21]. However, the mechanical properties of the structure limited its application in the high loading avenue. Two enhancement methods to improve its mechanical properties are noted, namely sintering and fibrosis [1, 8, 21] (these two methods improve the mechanical properties of the copolymer by changing the degree of polymer orientation). Similar to PLA and PGA, the co-polymer can also be manufactured into porous tissue scaffolds.

PEG family polymer has gained wide attention and it is rising star as tissue scaffold material nowadays. It possesses strong water repellence [22, 70, 93]. With certain functionalization, growth

factors and proteins receptors can be easily attached to the backbone of the hydrogel. On top of that, the polymer drainages can stop antigens and other undesired proteins adhering to the PEG scaffold surface, reducing the chance of negative immune response [65, 66]. PEG also has high compression factor due to its high molecular weight [94]. PEG and PEG copolymers such as PEG-PLA, PEGDA and PEG-PFF, all have excellent degradation rate and good biocompatibility [70]. More importantly, it can be easily synthetic by photo polymerization reaction. The figure 2-2 shows the comparison of natural polymeric material and artificial polymeric material regarding their advantages, disadvantages and fabrication method.

Natural Polymeric Material					Artificial Polymeric Material				
	Advantage	Disadvantage	Fabrication Method	Ref.		Advantage	Disadvantage	Fabrication Method	Ref.
Collagen	Biocompatible, natural ECM material	Immune reaction, poor mechanical property	Lyophilized, freeze-drying	[1,3,5,9]	PGA	Adjustable degradation rate, biocompatible	Poor mechanical property, hard to pattern	Compression and heating	[1,13-15]
Alginate	Biocompatible, photo-pattenable	Fast-degradation rate, poor mechanical property	Photo-patterning	[1,3,73,82]	PLA	Adjustable degradation rate, biocompatible	Low tensile strength and elasticity, hard to pattern	Compression and heating	[1,13-15]
Agarose	Biocompatible, can be processed as hydrogel	Fast-degradation rate, poor mechanical property	Injection	[1]	TCP	Self-curing ability, good biocompatibility	Hard to pattern, poor mechanical property, low load bearing ability	Heating and sintering	[15,18]
Fibrin colloid	From donor's own fibrinogen	Only useful to guide cell migration	Curing	[1,69,83]	PEGDA	Biocompatible, tunable stiffness, pattenable, controllable degradation rate	Need to attach adhesion proteins	Photo-patterning	[22, 65, 90]
Hyaluronic acid	Biocompatible, nature ECM material	Poor mechanical property	Injection	[15]	PCL	Biocompatible, high mechanical property	Hard to control the degradation rate, hard to pattern	Electro-spinning	[1,8]

Figure 2-2 Comparison of natural polymeric material and artificial polymeric material regarding their advantages, disadvantages and fabrication method.

There are various requirements need to be considered when we are selecting the fabrication material for 3D cell culture scaffold in this project. Firstly, it should be photo-crosslinkable, with elastic modulus tunable at fabrication between 0 and around 100 KPa [5]. Secondly, as discussed in

the introduction chapter, it should be able to include adhesion proteins and possess the ability for cell attachment. Thirdly, it should possess the ability to be made locally degradable by migrating cells. (For example, using matrix metalloproteinase (MMps) in the case of collagen, or by hyaluronidase in the case of HA) [14]. Nowadays, the most extensively investigated hydrogels by researchers in tissue engineering applications include: collagen, hyaluronic acid, PEG Acrylate/Diacrylate family, PVA, madrigals, etc. [15]. Figure 2-2 below presents the comparison of different hydrogels with regard to their biological properties and purchasing price.

	Possible initiators	Cell-degradable?	Cell adhesion	\$
PEGDA (unmodified)	Ciba Irgacure 2959 or Eosin Y	—	—	+++
PEGDA (modified)		+	+	—
Methacrylate gelatin		+	+	++
Methacrylated hyaluronic acid		?	?	+
Collagen	Riboflavin	+	+	?

Figure 2-3 Comparison of different hydrogel with regard to bio-property and price.

Collagen is a natural, biologically relevant material in which cells can migrate. However, even though photo-crosslinking has been demonstrated by Rose Bengal using riboflavin as photo initiators [95], the processes are not extensively characterized and the crosslinking illumination behavior has not yet been demonstrated to be precisely controllable. Hyaluronic acid (HA) is a key component of ECM and methacrylate HA photo crosslinking is a widely known method with highly controllable stiffness. Also, it has been proved that cells can migrate in it if they secrete hyaluronidase [15]. Gerecht and Marklein [96] also proved that it could be a suitable microenvironment for human embryonic stem cell self-renewal and differentiation. PEG family has shown well-characterized photo-crosslinking behavior, and its gel stiffness can be readily controlled. Also, synthetic collagenase-sensitive protein

has been integrated with PEG to allow cell migration. Cell adhesion proteins can be locally grafted to guide cell migration. More importantly, it is easy to be made photodegradable [5].

2.2.3 Photo Polymerization

Polymer is composed of a huge number of small molecules. Polymerization is the process which links all the small molecules together [97]. Even though the specific polymerization mechanism has not been fully understood, based on the general reaction process, polymerization can be classified into two kinds: step polymerization and chain polymerization [92, 97]. Chain polymerization and step polymerization differs in multiple ways. The most significant difference is the way in which the polymer is formed [64]. Specifically, for step polymerization, the reaction among the reactants happens stepwise. Firstly, one monomer combines with another monomer, forming a dimer, then the dimer combines with another monomer to form a trimer, and the process goes on until no monomers are available, resulting in large linked macromolecules [97]. The weight and size of the polymer molecules grow at a relatively slow pace [98]. However, for chain polymerization, an initiator is needed for the reaction process. The initiator releases the active species (the active species can either be free radical, cation or anion) which will propagate to bind a large number of monomer molecules in a chain reaction way. During this process, there is no reaction between the monomers, thus deeming the reaction more controllable [99]. Photo initiated polymerization is one typical chain polymerization. Photolysis of the chemical compound happens after exposed to certain wavelength light irradiation, breaking the chemical compound, resulting in free radical generation. The activated free molecular radicals rebind to each other and form a polymer chain until a mesh structure is formed in the end [97].

Overall, considering photo-crosslinking behavior and controllability as desirable attributes, the PEG family is considered the most desirable monomer choice. Previously, Chen et al. [17] utilized

PEGDA polymerization with their DMD based SL system and successfully obtained the woodpile structure, which shows promising utilization for the 3D printing system in this research. Based on their report, the same chemicals were chosen in our ultra-fast 3D hydrogel scaffold printing system. Specifically, Poly (ethylene glycol) diacrylate (PEGDA) is the reaction monomer, Irgacure 2959 (I2959) is the photo initiator and 2,2,6,6, -Tetramethylpiperidine 1-oxy (TEMPO) works as the reaction speed controller (radical quencher) [17]. Figure 2-3 below shows the chemical composition and the crosslinking process of PEGDA with photo initiator I2959.

Figure 2-4 Chemical composition and photo-crosslinking process of PEGDA monomer with photo initiator I2959.

2.2.4 Surface Nano-Scale Mechanical Property

Cellular behaviors depend strongly on their nano and micro-scale mechanical environment [100]. The artificial tissue scaffolds provide temporary mechanical support for the cells within the defects during the tissue regeneration; meanwhile, proper nano-scale mechanical property maintains the original

function of the tissue. For instance, nano-scale surface stiffness helps determine stem cells differentiate through mechanotransduction and matrix production [101, 102]. Proper gradients of the micro scale stiffness triggers stem cells migration (durotaxis) [100]. Gene expression of interacting cells is affected by nano- scale mechanical property and topography of the scaffold. Previously, Engler et al. [102] proved that the human mesenchymal stem cell differentiation is mediated by substrate nano-scale mechanical property: higher nano-elasticity of the scaffold could lead to cell differentiation into specific lineages and promote cells to commit to phenotypes such as neurogenic, myogenic and osteogenic. Ingber et al. [103] proved nano-scale mechanical property of ECM plays an important role in the procedure that embryogenesis changes to pathogeneissi (disease states). Therefore, it is essential that the nano-scale mechanical property of the fabricated scaffold should exhibit similar stress-strain responses as compared to nature tissues. The figure below shows the comparison of stiffness of different tissue and artificial polymer materials that have been used in tissue engineering.

Figure 2-5 Comparison of the mechanical property of the natural tissues and artificial polymers [5].

Previously, researchers mostly utilize tensile, compressive, dynamic mechanical testing and razor blade testing methods to characterize the elasticity or viscoelasticity property of polymer material [100]. However, these methods can only test material properties at macro level. On top of that, these traditional testing methods are only suitable for samples with higher elasticity: samples are usually tear or compressed, resulting in destroyed or useless samples after testing [104]. However, for soft polymeric material such as hydrogel PEGDA, final mechanical properties of the crosslinked product depend on its crosslinking density. Less crosslinked PEGDA leads to very fragile and gel state products [100]. Thus, it is difficult and inaccurate to use these traditional methods to test mechanical property of the PEGDA scaffold samples with low crosslinking rate and low elasticity. Furthermore, conventional testing methods are only suitable for testing polymer samples in dry state. However, hydrogel scaffolds are usually used in hydrated environment (cell culture medium) for tissue engineering application. Therefore, it is also important to know the mechanical property of the material under hydrated state. Overall speaking, these applications all call a technique that could measure the property of fabricated scaffold at the nanometer range that the cells interact.

Nano-indentation has become a tremendous useful tool for hydrogel scaffold surface nano-scale mechanical property testing [100]. It applies nano-newton force to the samples and receives displacement in nanometers, making measuring and testing the nano-mechanical property of tissue and polymers (especially soft material) accurately realistic. By controlling the indentation depth, researchers can conduct non-destructive testing for soft polymers [105] and change the material property accordingly to control the cellular interactions to match varied applications they are applied to.

Nano-indentation usually utilizes cantilever and laser-detecting method to conduct the force measurement [106]. The cantilever is very sensitive to the force: one end of the cantilever is fixed while a micro tip is mounted on the other end. During the test, incident light contacts between the

micro tip and the surface of the sample. Due to the presence of weak repulsive force (Van der Waals forces) between the tip atoms and sample surface atoms, the cantilever will move in a direction perpendicular to the surface of the sample with the change of the surface repulsive force [105, 107]. With the aid of optical detecting method, the position change and movement of the cantilever at different scanning point can be recorded, thereby, obtaining the sample surface topography information [100, 106]. The figure below shows the FESEM picture of the tip utilized in this research.

Figure 2-6 FESEM picture of the topography of the cantilever tip [106].

2.3 Scaffold Fabrication Techniques

2.3.1 3D Printing

3D printing has already found its application in the field of engineering, art, and manufacturing. Nowadays its tremendous advantages of usage in the field of biology science have also been rapidly recognized [33]. 3D printing of the scaffold for modeling human organs is a new strategy for *in vitro* tissue engineering study. 3D printer is able to print complex structure with high definition, making the modeling of the real organ realistic [108].

3D printing, also known as additive manufacturing, is defined as the process of joining materials to make objects from 3D model data, usually layer upon layer, as opposed to subtractive manufacturing methodologies (ASTM) (ASTM F2792). The 3D printing process, based on defined process, involved 2 main processes: design modelling and design production. The first process is the use of computer aid design (CAD) or other commercial engineering drawing software for 3D object model construction [109, 110]. The model file will then be saved in .STL format and transferred to the 3D printer. In the 3D printer, the .STL file will be sliced into a certain amount of 2D sequential cross-section slices depending on the resolution of the printer and finally the printer rebuilds the 3D objects layer-by-layer based on these 2D cross-sectional slices [69].

There are many different techniques for 3D printing available in the market, some are well established like stereo-lithography (SL) and fused deposition method (FDM) while others are up and coming like the electron beam melting (EBM) or bio-printing. There are a few factors in determining the machine of choice such as resolution, speed, materials available and build size. However most technologies involved with scaffold fabrication currently are using photopolymer resin as their materials. These include SLA, digital light processing (DLP), and two-photon polymerization (2PP).

Among all these techniques, the most widely adopted and highly commercialized 3D printing system for scaffold fabrication is SL. The approaches to the SL vary, but generally could be categorized into two main protocols: direct laser printing and mask based indirect printing [2, 74]. For direct laser printing, the single laser directly scans over the targeted area in the reaction resin to initiate the curing process. Once the curing of the layer is completed, the moveable stage in the resin carrying the cured resin sink by a small distance allowing new resin to cover the cured layer completely [68, 69]. This small stage movement distance determines the resolution. The laser then scans the new layer and hardens it. This whole process repeats itself until the end of the printing procedure. The direct laser printing technique provides advantages such as detailed feature fabrication as the laser scans

directly and moves with regard to the working stage as illustrated in figure 2-6 (left) below. However, the total process for direct laser printing is time consuming [33, 34].

To combat the problem of low productivity, researchers have further developed the conventional 3D printing to improve its productivity of 3D. Digital micro mirror device-based projection printing (DMD-PP) system is a good example for productivity improvement, shown in the figure 2-6 (right) below. DMD-PP technology is a projection system that has controllable digital mirror, which can reflect the laser light in an entire plane, enabling the curing of the entire layer at one time. The key device is the DMD that produces the image [34]. To build a part, the STL file is sliced and the sliced layer is converted into a bitmap file. The bitmap image consists of black and white regions where the white represents the material and black represents the void. When the image is projected onto the resin, the illuminated white portion will cure the resin while the black area will not. Once the layer is cured the build platform raises vertically upwards. This allows the machine to print parts without height restriction. However, resins with high viscosities may affect the lifting process. The process is completed when the entire layer have been printed. Unlike the SL systems, DMP-PP uses a mask projection for photo-polymerization and the building of structure is bottom up. Unlike the SL, which requires the movement of the stage as well as the laser, by integrating the convex lens, the large area fabrication time is largely reduced. However, beside the improvement for the productivity, the resolution of the printable feature is reduced.

Figure 2-7 Schematic of stereo lithography (two approaches). Left: direct laser printing; Right: DMD employed indirect printing [5].

Beyond the improvement for the system productivity, other systems aiming to improve the printable structure resolution have been developed by researchers as well [5, 34]. Maruo and Ikuta [111] reported a novel SL micro-fabrication method with single photon polymerization which enables them to narrow down the resolution in the depth to $5\ \mu m$. Comparing to the traditional stereolithography, which normally fabricates 3D structure layer by layer with the resolution of the final structure limited by the capability of the system, the Super-IH system they reported boasts enormous advantages over the traditional system with a highly precise pinpoint solidification function. [112].

Two-photon polymerization (2PP) uses femtosecond laser pulses that directly write the pattern into the volume of photosensitive resin. The 2PP process is similar to the SL in which light triggers a chemical reaction leading the polymerization of the photosensitive resin [66, 113, 114]. Majority of the material are transparent in the near infrared and highly absorptive in the UV spectral range. For the SL, the polymerization process takes place near the surface of a photosensitive resin due to single photo polymerization. As a result it is only possible to build 3D structures layer by layer. However, 2PP focuses the laser allowing fabrication of any desired polymeric 3D pattern by directly recording

or writing into the volume of photosensitive materials [72]. A simplified diagram is shown below to illustrate the difference between the excitation volume for linear absorption and two-photon absorption process.

Figure 2-8 Schematic of the difference between the excitation volume for linear absorption and two-photon absorption [115].

Due to the threshold behavior and nonlinear nature of the 2PP process, resolution beyond the diffraction limit of the optics used to focus the laser beam can be realized by controlling the laser pulse energy and the number of applied pulses [72]. The advantages of 2PP include better resolution spot size and the reduced need of an inert gas atmosphere. However due to the tracing method, the fabrication speed is slower compare to SL. Despite 2PP being a relatively new technology, its applications areas are rapidly expanding. 2PP are used for micromechanical systems, microfluidic devices, biomedical devices and scaffold fabrications for tissue engineering [66, 113, 114].

Nowadays, with further improvements such as galvanometer-based vector scanning [65, 116], SL systems has become highly commercialized and most widely used 3D printing machine [65, 109, 117]. SL can produce high-resolution products while the usage of the liquid medium is relatively low.

Also, the SL printer is designed to be smaller, faster, cheaper and simpler in terms of processing, aiming for future personal use [65, 109, 110]. An example will be the “Form1+” type high-resolution 3D printer developed by the ThorLabs Company. This personal desktop SL model is able to achieve industrial precision standards, at a more affordable price. It is able to print layers of up to 25 μm with a minimum feature size as low as 300 μm . The minimum 10 μm movement of the laser beam during scanning of the methacrylate photopolymer resin allows final products with smooth surface finish [109].

Other 3D printing technologies that can be considered to make scaffolds are fused deposition modelling (FDM), inkjet printing and bio-printing. The principles of FDM are based on surface chemistry, thermal energy and layer manufacturing technology [110]. Thermoplastic materials are melted by a heating element into a semisolid form. It is then extruded out through the nozzle onto a stage layer by layer [69]. As the material is extruded, it cools and solidifies to form the model. Once the first layer has been done, the stage lower by one layer and the process is repeated. The 3D printing method is the cheapest among all the current methods and has immense potential in future home printer applications [68, 69]. However, between each laid down layers, there exists air spaces and fusion lines which affect the final resolution of the product [110, 116] .

Inkjet printing is a non-contact technique capable of reproducing digital image data on a substrate using picolitre droplets [2]. The technique is similar to how commercial inkjet printer works. However instead of ink, photo-resin or wax is jetted out. Jetting heads will release materials onto a tray and UV attached to the jetting head cures the material. Once the material is cured, the build tray will be lowered and the next layer is built [5]. The advantages of this system include high quality and accuracy; fast build speed and the ability to print multiple materials [2, 5].

Dispensing is described as the release of material continuously usually in filament form [2]. This includes extrusion. For extrusion, pressure is used to force the material through a nozzle in a

controlled manner to construct a 3D structure. Once material is deposited, solidification of the material through physical or chemical means provides sufficient mechanical integrity to fabricate 3D structures [6]. Depending on the printer, either the printer head or stage will move while dispensing the material to form the pattern. The materials used are usually highly viscous hydrogels [2].

Jetting is described as the release of material in droplet form for better precision. It uses either the inkjet head or micro valve technology [2]. This technique can be divided into two basic categories namely, continuous inkjet (CIJ) and micro valve printing. In CIJ, a steady stream of small droplets is produced when pressure oscillations are being applied to the stream. The droplets is either deflected by an electrostatic field onto a substrate or not deflected and collected for reuse [2, 5]. Drops of inkjet were produced when required. A volumetric change in the fluid initiates the droplet formation done either by thermal or piezoelectric effect. In thermal inkjet printing, rapid local heating generates a bubble within the ink chamber that ejects a small droplet while piezoelectric inkjet printing is used to create a pulse resulting in droplet ejection [2, 5, 6].

Overall, a major drawback of 3D printing is that it is a time consuming process as the layer-by-layer stacking of the material or laser scanning normally takes hours. Furthermore, the time needed for processing is largely dependent on the resolution required. The higher the resolution of the printing, the longer time will be needed for processing. With longer processing time, the cost of the fabrication will also increase. This drawback makes the high throughput manufacturing of tissue scaffold fabrication not possible [2].

2.3.2 Interference Lithography

The interference phenomenon can be described as the overlapping of multiple optical or electromagnetic waves [36, 37]. The simplest one-dimensional interference pattern can be generated by two coherent light waves, and the famous Young's double slit interference experiment is the best

example [36, 37]. The interference fringe pattern consists of periodic sequence stripes. These stripes represent the maximum strength and minimal strength. Figure 2-8 below shows the interference of two coherent laser beams and the interference pattern they create.

Figure 2-9 Schematic of interference of two coherent laser beams [37].

Overall, in two-beam or multiple beam interference, any point in the spatial could be established as the superposition of all the electric field at a certain time t [37].

$$E(r, t) = E_1(r, t) + E_2(r, t) + \cdots E_n(r, t) \quad (2-1)$$

The equation above demonstrates the intensity distribution of the superposition region of the electric field. Where, E_i represents the i^{th} electromagnetic wave. More specifically, the intensity distribution equation can also be further described by a Fourier superposition [37]:

$$I = \sum_i E_i^2 + \sum_{i < j} E_i * E_j \cos[(\mathbf{k}_i - \mathbf{k}_j) * \mathbf{r} + (\varphi_i - \varphi_j)] \quad (2-2)$$

Where, E_i represents the amplitude, k_i denotes the wave vector while φ represents the phase of the beam. The intensity distribution varies from point to point and the interference pattern generated also varies according to the difference of phase, amplitude, wave vector and polarization of these electric fields [41]. Thus, phase, along with amplitude, wave vector and polarization are important

laser beam interference properties [36]. 2D and 3D interference patterns can be manipulated by controlling these properties. Specifically, 2D dot arrays and 3D lattice patterns can be fabricated with three and four-beam interference [36, 37, 40, 118, 119] as shown in the figure below.

Figure 2-10 Periodicity that can be produced with different beam interferences [37].

Interference lithography (IL) is a technique that integrates the interference and diffraction characteristics of light. It regulates the intensity distribution of the light field through different combination of the beams, and records these intensity variations using photosensitive materials [36, 37, 40, 120-124]. The system for interference lithography does not require an expensive interferometer projection optical system, and it can fabricate patterns on a large scale, the only limitation for the exposure field being the system aperture [36, 37]. What is more, with the sub-micron IL technique, the resolution of the pattern could be further reduced to $\lambda/4 - \lambda/2$ [36, 37]. Conventional lithography is usually limited to a small exposure field to increase the resolution feature. In order to improve productivity, large area fabrication is usually conducted with scanning projection systems [37, 41]. These projection systems require complex work piece stage with precision positioning control, which

will greatly increase the cost of the lithography apparatus [37]. More importantly, traditional lithography system is unable to directly fabricate the 3D structures due to the limited focus depth [37, 41].

3D microstructures have varied application in the field of photonic crystals, biomimetic structures, optical communication, tissue engineering and etc. [36-38, 40, 123, 125-127]. Traditional multiple-beam single exposure interference lithography for the 3D microstructure construction has advantages over the fabrication time and process (one exposure) [37, 41]. In 1995, Mei et al. [128] firstly introduced the possibility of using multiple laser beam interference lithography to fabricate 3D structures. Later in 1997, Berger et al. [129] successfully created 2D pattern using three-beam interference in the SU-8 photoresist. Since then, different interference lithography systems and approaches have been developed to fabricate different 3D periodic structures [10, 36, 119, 123, 126]. Later, in 2000, Campbell et al. [40] demonstrated a solid 3D lattice structure by translating 3D light intensity distribution into a thick photoresist layer. Cai et al. [130] later on demonstrated all 14 kinds of Bravais lattices of light intensity distribution could be formed and manufactured by four non coplanar beams. One representative work comes from Moon et al. [123], they firstly integrated the wave plates in his IL system to induce phase shift for the laser beams [131]. They managed to fabricate 2D and 3D polymer patterns through manipulating the phase of the beams. One laser beam was initially incident on the wave plates, and the polarization of the beam in the fast axis of the wave plate was linear. Then the wave plate was rotated to the direction parallel to the polarization direction. The phase difference obtained from the plate rotation was π . The phase changes of the wave plate changes the interference pattern further. The 2D square lattice pattern was generated with three beam single exposure. The Face Centered Cubic (FCC) lattice 3D structure was fabricated with four-beam single exposure [123, 131].

However, the multiple-laser beam interference system requires precision optic alignments and

strict system stability [38, 42, 131, 132]. To overcome this drawback, prism has been utilized as the beam splitter in IL for fast and large area fabrication of quasi-crystal structures [36, 37, 118, 133]. The simple beam splitting process largely reduced the system vibration sensitivity. What is more, the multidirectional side surfaces of the prism can be the tool for producing multidirectional slanted structures [133]. However, a notable drawback of the prism is the limited exposure angel in the resin [37, 41]. One novel protocol to increase the exposure angel of the prism is brought up by Jiang et al. [133], he integrated the incident UV IL with a prism to deflect the light and enhance the exposure beam angle range with slanted stages. Figure 2-10 below shows their simple set up, the inclined angle was easily manipulated with varied slanted stages. They managed to fabricate slant structures with inclined angles ranging from 15 degree to 60 degree.

Figure 2-11 Schematic of integration of prism with slanted stages to enhance the light exposure angle [89].

Current research of IL has been focusing on expanding the control over the ultimate feature size, the structure complexity and the structure resolution [36, 37, 123]. The minimal produced feature size is proportional to the laser beam wavelength [37, 41], thus, one solution is to utilize shorter

wavelength laser light, for instance, utilizing the extreme ultraviolet laser and X-ray lasers [36, 37, 41, 123, 134-136]. Wang et al. [119] applied extreme ultraviolet (EUV) interference lithography to fabricate a six-fold symmetry Kagome lattice and they managed to control the phase shifts of the beams by placing a transmission diffraction gratings mask on the Electron Beam Lithography (EBL) system [119]. Feature sizes down to 40 nm and periods down to 180 nm Kagome lattice structures were fabricated. However, a notable problem is, these short wavelength regimes will rapidly change the optical constants due to the atomic absorption edge [134]. In order to eliminate the optical constant change, researchers have further improved the system. Mojarad et al. [134] firstly demonstrated utilizing a novel transmission-grating broadband mask for EUV cutting-edge resolution soft interference lithography. The broadband mask possesses high diffraction efficiency, which enhances the diffraction of the EUV. In a recent study by Brueck et al. [137], they took advantage of the anisotropic etching of Silicon by KOH and successfully reduced the pattern size down to a half-pitch (22 nm) with their ‘signal-exposure self-aligned spatial frequency doubling’ technology.

Another solution to improving feature resolution is by using of liquid immersion technology [36, 37, 126, 138-140]. Liquid immersion replaces the usual air gap between the photoresist and the incident laser beam with a high refractive index liquid medium [36, 37, 126, 138]. The resolution could be greatly improved with the enhanced refractive index as a result. Chang et al. [126] developed a new liquid immersion IL system based on the traditional Lloyd’s IL. Lloyd’s mirror IL utilizes a mirror to create a virtual light source, making rapid period changes easily achievable [36]. However, the feature size is limited due to the low ambient index of the air [36]. As a result, the smallest possible period to be achieved is around half of the incident light wavelength [126]. By integrating the high refractive index of immersion fluid, they managed to fabricate periodic gratings from 170 nm-112 nm with a 325 nm wavelength UV laser [126, 139-142]. However, the optical path lengths are not the same in the system. The different absorption rates of the two beams can result in the imbalance of the two interference beams, resulting in the pattern produced to have lower fringe contrast and line

width variation [36, 37, 126]. What is more, this method is only applicable to 2D grating fabrication and more complex 3D structure fabrication with high resolution has yet to be reported [126].

Another parameter to consider with interference lithography is the photoresist itself [36, 37]. Common photoresist such as SU-8 has higher absorption coefficient at short wavelengths [41]. On top of that, the evanescent wave decays from the surface of the medium exponentially [36, 41]. Therefore, low light intensity cannot efficiently transfer the interference patterns to the photoresist. In order to overcome this drawback, the photoresist films are always made thin enough to ensure structure integrity [36, 38, 125, 138]. Another possible solution is to enhance the incident light intensity. One novel solution to enhance the incident light intensity was brought up by Niu et al. [138], they utilized a high index prism coated with metallic film to enhance the light intensity and exposure depth of the interference lithography [138]. The triangle prism coated with metallic film was placed just above the photoresist, the polarized beam incident at the interface of the prim and the coated film at the resonance angle.

Another approach to interference lithography is mask-based holographic lithography [36, 41, 123, 127]. A 3D image is recreated after a single coherent optic beam is exposed to the hologram, and subsequently gets transferred to the photoresist. The idea of holography was first brought up and discovered by Hungary scientist Dennis Gabor when he was working to enhance the electron microscope resolution in 1947 [143, 144]. Back in the old times, the best collimated light source is the mercury arc lamp, therefore the holography technology was largely limited by the collimation of the light source. By 1960s, the invention of lasers brought breakthrough to the holography technology. The laser light beam is considered to be coherent, pure and intense making it very suitable for holography technology. In 1962, Emmett Leith and Juris Upatnieks repeated Gabor's work by using lasers and integrating their own "off-axis" technique. They finally managed to produce the first

transmission 3D hologram which can recreate the 3D image with laser exposure [143, 144]. Figure 2-11 shows the first 3D image with the holography technology.

Figure 2-12 First 3D reconstructed image produced by the researchers in 1964 [109].

The word hologram comes from Greek, which means translate something into whole picture [144]. Hologram is actually also a kind of photograph. However, the hologram photograph is different from the conventional photograph. For instance, the conventional photograph is just the 2D recording of the information on the plane, while the hologram consists the 3D information of a certain object. Different location on the hologram surface records different spatial image information [36, 41, 123, 127]. There is certain relationship between phase requirement at the hologram plane and the intensity distribution at the focal plane to create the image which means, upon illumination, the hologram could produce 3D images just like a projection machine in a 3D fashion [144]. Figure 2-12 below illustrates the working mechanism of the hologram, and the reconstruction of the object image with the laser beams.

Figure 2-13 The hologram of a 3D object can be considered as a superposition of many individual holograms of points on one object [110].

Holographic lithography and interference lithography are similar in the aspect of their system composition [41, 133]. Both of the lithography systems are composed of low Numerical Aperture (N.A) lenses, mirrors, prisms and dichroic mirrors. Neither of the systems requires the expensive high-resolution wide-field lens projection system employed in the traditional lithography system [38, 42, 127, 132]. The difference is that the former system utilizes a hologram to create the light interference while the latter uses several laser beams for interference. IL can only fabricate periodic structures while HL can produce generally any feature [38, 85, 87]. Holographic lithography possesses some advantages over IL in the aspect of system cost and system reliability [38, 42, 127, 132]. Interference lithography requires accurate system alignment due to different light paths and vibrational instability, which is not required in the HL system.

Phase mask is a surface relief grating with index variation in height direction, which could diffract incident laser beams accurately [36, 38, 42, 118, 127, 132]. It is the most widely used simple hologram in the HL field nowadays. The figure below shows the working principal of the 1D phase mask under one beam exposure. The phase mask divides the laser beam into three major stable output beams (first, second and zero order) once the beams passed through the phase mask, the output beams are overlapped in the photoresist region. Periodicity was therefore generated in both X and Z directions [36, 38, 42, 118, 127, 132].

Figure 2-14 Schematic of the working principle of 1D phase mask [36].

Phase mask has been utilized in holographic lithography to write gratings in fibers before [36]. Now researchers have found their application in the field of lithography for producing 3D microstructures in the photoresist. For the first time, Lin et al. [42] demonstrated utilizing phase mask instead of multiple-beam complex system to fabricate 3D structures. By double-exposure of the phase mask, the tetragonal and orthorhombic symmetries two-layer woodpile structure was fabricated in the SU-8 photoresist. Xu et al. [132] further produced a two-layer phase mask by integrating nano-imprinting and Polydimethylsiloxane (PDMS) mould casting method. The double-layer phase mask they produced managed to divide one incident laser beam into five diffractive beams, thus more complex multiple Face Centered Tetragonal (FCT) symmetric structure can be produced with single exposure. To our best knowledge, no work has been reported using more complex hologram to produce 3D structures other than using the phase mask. One possible reason is due to the complex hologram design and fabrication process [36].

2.4 Multi-functional Hydrogel for Tissue Engineering

Applications

Hydrogel refers to crosslinked water-soluble polymer chains formed in a 3D manner, which can be produced by the simple reaction of one or more monomers [64]. The hydrophilic functional group attached to the backbone of the polymer renders the hydrogel capable of absorbing a large amount of water within their 3D network [145]. Meanwhile, the crosslinked polymer chains in the network prevent the hydrogel from dissolution. Thus, the 3D network hydrogel usually possesses porous structures and these porous structures permit the effective immobilization of drugs or biomolecules into the hydrogel matrix and then release through the gel network mediated by the molecules diffusion coefficient [64, 101, 145]. By changing the hydrogel crosslinking density, their porosity and pore size can also be controlled. What is more, the photo-patternable and deformable ability of hydrogel enable them to conform to the shape of the application to which they are applied, enabling it to be engineered to mimic the composition and structure of the natural ECM. Furthermore, the tunable stiffness of the hydrogel can be simply achieved by altering the hydrogel crosslinking degree [146]. Overall, due to these tremendous advantages, hydrogel has seen its major applications in tissue engineering scaffold fabrication and drug delivery [61].

Previously, research surrounding hydrogel tissue scaffold design and fabrication mainly focus on choosing, synthesizing and tuning the hydrogel mechanical property, molecular weights and biodegradability to meet the needs for specific applications [7, 8, 14, 147]. The hydrogels were then fabricated into 3D architectures with different surface textures to mimic the natural ECM. Based on this, varied synthetic polymers have been utilized for fabrication tissue scaffold [148-150]. Poly (N-isopropylacrylamide) (PNIPAm), 2-hydroxyethyl methacrylate (HEMA), 2-hydroxypropyl methacrylate (HPMA), acrylamide (AAm) and PEG etc. are some of the polymers frequently studied.

Researchers have utilized HMPA, HEMA and AAm to fabricate scaffold to mimic bone and cartilage tissues due to the non-biodegradability of the polymer [147]. PNIPAm has been utilized to detach cell layers to fabricate special tissue such as cornea or cell sheets due to their temperature-responsive swelling property, the polymer undergoes phase separation and de-swelling when the solution temperature is higher than 32 degree [147]. PEG-based hydrogel is one of the most extensively investigated polymers due to the advantages that have been stated in the previous chapters. However, PEG hydrogel is neither cell adhesive nor biodegradable, largely reducing its ability to provide an ideal environment for cell growth [150]. As mentioned before, tissue regeneration process usually accompanies three phases: cell proliferation, cell differentiation and tissue morphogenesis. Thus, tissue scaffold should not only provide stable structural support, desired mechanical property and high porosity, but also, more importantly, possess the ability to promote cellular functions. Simple PEG hydrogel scaffold without modification or functionalization cannot meet the requirements for cell proliferation and differentiation for further tissue regenerative purpose [4].

Thus, creating functional hydrogel with improved bio-activeness to satisfy diverse requirements in tissue engineering applications has become a new focus in recent studies [151]. Although PEG is not cell adhesive, it can be easily modified with various functional groups such as carboxyl, thiol, acrylate or biomolecules (such as cell adhesive peptides, enzyme peptides and growth factors) due to the hydroxyl group at the end of the backbone [147]. For instance, researchers have modified the PEGDA with cyclic cell adhesive peptides cRGD and found the cell adhesion activity was enhanced up to 240 times [147]. Meanwhile, enzyme-sensitive peptides (ESP) were also incorporated with PEGDA in order to mimic the degradation mechanisms of proteolytic that occurred in natural ECM. What is more, growth factors (GFs) are polypeptides that play significant role in regulating cellular activities. They bind to specific receptors on the target cell surface to mediate cell functions and tissue regeneration [152]. Researchers tried to modify GFs in order to attach the GF to the PEGDA backbones [150]. Hubbell et al. [19, 149] investigated a new kind of VEGF with cysteine

and successfully attached the GFs to the PEGDA. They also engineered VEGF-modified with Gln acceptor peptide, which can be attached to the Kys Donor peptide (KDP) modified PEG hydrogel. West et al. [148] engineered covalently bonded acrylated GFs, which can be easily attached to PEG hydrogel. Jabbari et al. [153] incorporated morphogenetic protein (BMP) derived peptide with PEG hydrogel network and showed a positive effect on promoting the osteogenic differentiation of bone marrow cells. However, such modification is usually time consuming. Also, GFs or varied peptides may lose their functionality after the modifications, as most of them are fairly sensitive. The easiest yet most effective way to incorporate the biomolecules into hydrogels is still directly loading these agents into hydrogel before crosslinking. However, such method usually suffers from burst release during the initial hydrogel swelling stage [147].

Meanwhile, a new trend of multi-functional hydrogel scaffold has drawn wide attention in the research field nowadays. Such hydrogel scaffold integrates controllable drug delivery technology with scaffold fabrication, making hydrogel scaffold a 3D structural support for the cells and therapeutic drug/biomolecule carrier at the same time. It shows a convenient way to deliver bioactive molecules to the cells without further complex functionalization of either the scaffold or the molecules. More importantly, the localized drug delivery prevents the side effect and risk of overdoses during systemic administration, and enables the target tissue to access to a higher concentration of medication [154]. Laurent et al. [155] incorporated high concentration of doxorubicin and 5-fluorouracil in the 3D hyaluronan (HA) scaffold in order to test the inhibition effect of the drug carrier/scaffold for cancer cell treatment. Such scaffold provided suitable 3D environment to investigate the localized drug delivery effect on the cancer cells. Thakur et al. [156] fabricated a drug carrier/3D scaffold by incorporating antibiotic mupirocin and lidocaine poly-L-lactic acid (PLLA) for wound healing test, the multi-functional scaffold not only provided a 3D platform for the cell attachments at the wound site, but also, the controllable release of two kinds of drugs prevents the wound inflammation and fastens the wound healing process. Lee et al. [157] loaded growth factors in the alginate hydrogels scaffold.

The scaffold could react to the mechanically stressed environment and release the GFs accordingly. Afterward, they applied the scaffold to *in vivo* animal model and found the cell proliferation rate and tissue regeneration were greatly promoted with the application of external mechanical signal. Wang et al. [154] engineered microsphere growth factors into polymer scaffold and applied the scaffold to human bone-derived mesenchymal stem cells. Multiple growth factors delivered from the scaffold significantly promoted the stem cell differentiation. Sohler et al. [158] engineered myoglobin and lysozyme proteins into three layer poly (ether-ester) multi-block copolymer scaffold, the successful release of the protein from the scaffold significantly benefits the cell attachment. Shi et al. [159] loaded drugs and protein into poly (lactic-co-glycolic acid) (PLGA), the drug carrier/scaffold showed an excellent positive effect on calcium secretion and MSC proliferation. In conclusion, conventional hydrogel scaffold can no longer meet the advance of tissue engineering studies, functional hydrogel scaffold with therapeutic drug delivery ability has seen a new era.

2.4.1 Hydrogel Particle Delivery Mechanism

The main obstacle of designing functional tissue scaffold as therapeutic drug carrier is to achieve controllable release of encapsulated agents from the tissue construct (to provide critical cures for cell immigration, upregulation of the ECM or cell regeneration), and also at the same time, to prevent the excessive leakage of the drug (which may induce local toxicity and result in sever systemic immune reaction of the patients). The release mechanism of the hydrogel drug delivery system depends on two main factors: the method the drug is incorporated to the hydrogel matrix and the physiochemical properties of the hydrogel network [145]. Specifically, two methods are widely utilized for the incorporation of drugs into the hydrogel. (1) Post-loading: hydrogel network is firstly formed by polymerization followed by the absorption of the drug. For this method, the main factor that may affect the release of the drug is drug-hydrogel interaction and the drug diffusion; (2) *in-situ* loading: drugs are encapsulated with the hydrogel before polymerization. For this method, the release of the

drug is mainly affected by hydrogel swelling, degradation of the hydrogel and the diffusion of the drug particles [64].

Diffusion controlled delivery system

Diffusion controlled release system relies on the high permeability and porous structure of the hydrogel. Drugs are time-independently and constantly released (through diffusing behavior) from the mesh structure or the water filled pores to the hydrogel membrane then to the external surroundings. Diffusion coefficient of the drug particle (which relates to the pore size and tortuosity of the hydrogel) is the main parameter to be considered. The one dimensional drug release of this kind of system can be described using Stefan-Maxwell equations or Fick's law [145]:

$$J_A = -D \frac{dC_A}{dx} \quad (2-3)$$

Where J_A is the molar flux of the drug (mol/cm^2); D is the diffusion coefficient of the drug in the hydrogel; C_A represents the drug concentration. In most cases the drug are considered uniformly distributed in the hydrogel and the diffusion coefficient is considered to be constant through out the releasing process. For steady state diffusion, the drug release can be further described as [145]:

$$J_A = K \left(\frac{D \Delta C_A}{\delta} \right) \quad (2-4)$$

Where K is the ratio of drug concentration in the gel, δ is the thickness of the hydrogel matrix. While for the drug particles are uniformly distributed in the 3D hydrogel matrix with unsteady diffusion, the release function can be defined as Fick's second law [145]:

$$\frac{dC_A}{dt} = D \frac{d^2 C_A}{dx^2} \quad (2-5)$$

The diffusion coefficient is also considered as constant.

Swelling controlled delivery system

Swelling controlled systems utilize glassy polymer matrix for drug encapsulation. The hydrogel may undergo glassy transition phase once it encounters biological fluid. Then the macromolecular chains of the hydrogel relax, resulting in the rapid diffusions of the entrapped drugs through these swollen rubbery to the surroundings. For instance, hydroxypropyl methylcellulose (HPMC) loaded drug utilizes its swelling property for moderate drug release: as the polymer absorbs water after oral dosing resulting in controllable drug diffusing modulated by the water absorption ratio. The drug release ratio is mainly determined by two factors, namely the diffusion time and the polymer chain relaxation time, which can be described as the empirical power law [62, 145, 160]:

$$\frac{M_t}{M_r} = k_1 t^m + k_2 t^{2m} \quad (2-6)$$

Where k_1, k_2, m are all constant numbers. M_t is the amounts of drug released at time t and M_r is the drug released at equilibrium.

Chemically controlled delivery system

Chemically controlled systems utilize the reactions occurring within a matrix to deliver the molecules. One of the most common reactions within hydrogel system is the dissolution or degradation of the polymer chains. The particles binding to the polymer chains have release rates mediated by the binding equilibrium. Under certain circumstances, the bulk erosion of the hydrogel backbones or degradation of the polymer chains will result in the final release behaviour [145].

2.4.2 Bioelectricity

The human body is governed by various biophysical and biochemical stimuli (such as chemical, mechanical, material and magnetic etc.) to maintain the structural and functional stability [161].

Nowadays researchers are exploring the possibility to apply these stimuli to *in vitro* and *in vivo* models to further control and improve the tissue implants. Among all the factors, electric stimulation has been an active topic in recent tissue engineering and drug delivery researches, varied studies have proved electric field not only has strong effect on the functioning of nerves and muscles but also plays a significant role in the maintenance of cellular functions, tissue scaffold morphologies, cell proliferation, cell migration, gene expression and wound healing [161-170].

Bioelectrical phenomena are widely found *in vivo* tissues and cells. There are electric gradients in biological tissues and cells, especially among nerve, muscle, neural crest, fibroblasts and epithelial cells [50, 67, 171]. For instance, 5 to 6 mV positive potential difference is found between the back of the eye and the eye cornea [172], tens of millivolts electric gradient are found across the nerve cell membrane. The electric gradients across the membrane of the cells are called membrane potential, it is the main source of *in vivo* bioelectricity [161, 173]. The size and polarity of the potential are mainly determined by three factors namely the intracellular and extracellular ion species such as K^+ and Ca^{2+} , the ion concentration and the membrane permeability [53]. Varied kinds of proteins such as ion channels, enzymes, protein receptors and transporters on the membranes can sense the voltage change in the extracellular and intracellular fluid, and then regulate the cellular functions accordingly [79, 95, 169, 174, 175]. The difference of ion concentration across the membrane could activate the signaling pathways on the membrane. The signal transduction across the membrane conducts when the potential difference reaches in the order of 100 mV [164, 173]. The change of the trans-membrane potential causes activation of the channel-mediated ions such as K^+ and Ca^{2+} , resulting in the rearrangement of the protein receptors through polarization of the membrane [174]. The skeleton of the cell keeps the integrity of the whole structure during the series of alternation. The intracellular electric field triggers the adenosine triphosphate (ATP) depletion and modifies the activeness of certain linker proteins on the skeleton such as spectrin and ezrin/ radixin/ moesin (ERM). This series of deformation cause different cell behaviors such as adhesion or migration [162, 169, 176, 177]. However, the mechanism

behind this phenomenon is still under study and whether Ca^{2+} is of dominating effect in the process is still debated.

During the cell regeneration and embryonic development, the polarization plays an important role in regulating the cell behavior and triggering the self-spatial patterning of the cells [67]. Interruption of the electric gradient and polarization in embryos could result in severe results such as reduction of regeneration of the new cells, healing process completely blocked or even loss of eyes [53, 163, 168, 178]. The effect of electrically induced cell migration and self-spatial patterning regulation is called galvanotaxis or electrotaxis [176, 179, 180]. Li et al. [162] firstly discovered the phenomenon when he applied electric stimulation to the HT-1080 fibrosarcoma cells. It was found cells always migrated to the cathode side; meanwhile, the concentration of reactive oxygen species (ROS) within the cell was also largely reduced. ROS is a kind of chemically reactive molecule that regulates various cell behaviors. Excessive ROS may lead to cell damage, aging or even death. Low concentration of the ROS particles could promote the cell proliferation, cell division, cell migration, gene expression and signal transition [56, 168]. They also found the extracellular signal-regulated kinase (ERK) signaling pathways and the trans-membrane receptors were more active with lower ROS molecules under the electric field. Serena et al. [181] also discovered, under the effect of electric stimulation, the concentration of endogenous ROS of embryonic stem cell was reduced, enhancing the cardiac differentiation. Yuan et al. [182] found out when the endothelial cells were subjected to electric stimulation, the expression of EDN1, PDGFA, PDGFB and adenosine A_{2b} genes were largely increased. These genes are the key encoding angiogenic factors modulate the behavior of chondrocytes and meniscus cells, enhancing the meniscus cell migration [52, 56, 71, 162, 183].

Bioelectricity also has a strong influence on the gap junctions. Gap junction is the protein oligomers contact region on the membrane of two adjacent cells. Researchers have found there are 2-4 nm junctions between the neighboring membranes of the osteoblasts cells [166, 172, 178]. Gap

junction regulates the propagation of ion nutrients, metabolites, and small molecules between cellular interactions. It provides communication channels for delivering signal molecules such as cyclic nucleotide and inositol phosphates between two adjacent cells. Specifically, these signal molecules may further control the differentiation of osteoblast cells, dividing the cell into different phenotypes, such as alkaline phosphatase, bone sialoprotein, collagen and osteocalcin. Nakagawa et al. [184] proved when different voltages were applied to the gap junction protein C*26, it tended to selectively deliver small molecules. Lohmann et al. [166] found out the gap junction protein C*34 were largely reduced in MLO-Y4 cells and ROS 17 / 2.8 cells under low frequency electric field. Nishizawa et al. [69] discovered, when myocytes cells were applied to electrical stimulation with amplitude 10 mA, the delivery of the Ca^{2+} ions were observed to dramatically increase in the gap junction.

Another strong proof for the electric gradient across the cell membrane is the trans-epithelial potential (TEP), which can be widely found in the injured of epithelium. The small current loop flow along the two sides of the epithelium wound (around 20-40 mA / cm^2) controls the division of the new cells and directs the cell migration towards the injuries [177]. This TEP dominates the effect during the wound healing process, which may override the influence of other stimuli such as chemical gradients, scaffold mechanical properties and population pressure [53, 168]. Baker's group [185] confirmed the influence of the bioelectricity in the human regeneration in bones, which is similar to the mechanism of TEP healing. They utilized streaming potential, streaming currents and piezoelectricity of collagen molecules to mimic the bioelectricity in bones, they found out continues electric current at the wound part of amputated fingertip of children could stimulate the gene expression of transforming growth factor- β (TGF- β), collagen type-I, alkaline phosphatase (ALP), bone morphogenetic proteins (BMPs) and chondrocyte matrix. While TGF- β 1 is the main factor to regulate osteochondral cell proliferation and differentiation, promoting the extracellular matrix deposition, enabling fast regeneration of the new bone cells. Meng et al. [178] also confirmed that

under electric stimulation condition, the gene expression of ALP, Runx-2 and BMP2 of osteoblast cells is double the amount of the cells without treatment.

Thus, due to the broad influence of bioelectric phenomenon in human body, developing electric-stimuli responsive biomaterial with easy to tailor property for either tissue scaffold fabrication or ‘smart’ action drug release is a highly desired goal for tissue engineering studies.

2.4.3 Conductive Hydrogel for Tissue Engineering Application

The electrical stimuli-responsive hydrogel is also called “smart” biomaterial, as it could change their volume or release of substances once an external signal is applied [186]. It holds great potential for next generation bio application such as bio-electronic sensors, tissue engineering and drug delivery due to their low-cost fabrication, design flexibility and superior performance stability [187]. Most importantly, electro-conductive hydrogel exhibits a major advantage as a drug delivery material in that it has a fast drug release response upon electric influence, which is better than the conventional drug delivery methods such as oral dosing and transdermal drug delivery [48]. Structurally, the electro-conductive hydrogel networks are composed of hydrated 3D structures doped with conductive materials that impart the electronic conductivity feature throughout the whole material. The composite material usually exhibits combination properties of their components, such as the elastic behavior, flexibility, softness, biocompatibility of the hydrogel and the electric conductivity, which could be easily driven by an external signal, and high stiffness of the conjugated conductive materials [46-48, 63]. By varying their material concentration or crosslinking ratio, the chemical and mechanical properties of these materials could be easily controlled.

The first reported electro conductive hydrogel work was from Gilmore’s group [47], they incorporated polypyrrole (PPy) with polyacrylamide hydrogel. The resultant composite hydrogel

retained its hydration and at the same time showed electro conductivity. This pioneering work has paved the path for conductive hydrogel study. Based on the novel idea, researchers further explored the use of conductive hydrogel in tissue engineering applications such as in building conductive tissue scaffold and electric-assisted drug release. Shin et al. [188] for the first time incorporated GO as the reinforcement material into gelatin methacrylate (GelMA) and created cell-laden graphene-embedded hydrogel. The reinforced GO largely enhanced the electrical property of the hybrid scaffold and made the material mechanical strength tunable while the mechanical durability and stability were also increased. The improved cellular performance was observed in *in vitro* experiments where the hybrid polymer showed excellent traits as a tissue scaffold material. However, the main focus was limited to the improvements of the mechanical properties of the hydrogel scaffold. The performance of the conductivity of the scaffold and its effects were not explored or quantified. Liao et al. [189] also utilized GO as the supplementary material in their methacrylated chondroitin sulfate (CSMA) and poly (ethylene glycol) methyl ether-caprolactone-acryloyl chloride (MPEG-PCL-AC) scaffold for cartilage tissue engineering. The overall mechanical and electrical properties of the scaffold were reportedly greatly improved. They stated that the electric conductivity of their designed scaffold could potentially benefit the signal transduction between cells. Li et al. [190] synthesized 3D graphene foam scaffold using chemical vapor deposition method for the first time. Their new trend of scaffold involved a conductive platform utilized to introduce external electric stimuli for *in vitro* neural cell studies. They found that the material has a superior advantage in electrical, chemical and thermal properties. The large specific surface area of the 3D graphene promotes the charge injection ability, and after applying electric stimuli to the scaffold, neural cell tend to be more active. Up-regulation of Ki67 genes expression was also observed, indicating that the 3D conductive graphene scaffold enhances cell differentiation and proliferation.

There are also studies on the development of electric-assisted drug delivery technologies with different material and incorporation methods. For instance, Kwon's group [191] have developed

conductive polyelectrolyte hydrogels, which is pH sensitive. Upon current induction, they observed an increased in the local pH, which consequently stimulated the swelling and de-swelling behaviors of the hydrogel. These behaviors further induced morphology changes of the hydrogel, which potentially cause erosion. Pillay et al. [160] reported electric stimuli assisted “ON-OFF” drug release method using polyaniline and poly (vinyl alcohol) based hydrogel. They reported that the conductivity of the doped polymer increased linearly with the increase of polyaniline additives. They also observed electro-actuation (erosion of the hydrogel) after the electricity was applied. In another study, Luiz et al. [192] interpenetrated polyaniline chain inside the polyacrylamide pores and the copolymer showed increased electric activity. The relative larger pore size of polyacrylamide provided space for storing volumes molecules, and the molecules were liberated with electrochemical reactions. Madou et al. [193] incorporated poly (2-hydroxy-ethylmethacrylate) –poly (N-vinylpyrrolidone) with PANI and created the copolymer which may undergo shrink and swell under specific electrical currents. Niamlang et al. [194] developed drug doped poly (phenylene vinylene) / PAAm hydrogel which could release drugs under electric field. Jun et al. [187] incorporated drugs with conductive nanoparticle polypyrrole (PPy), and then further encapsulated with temperature sensitive hydrogel. The drugs were released from PPy nanoparticles, once the electric field was applied, towards the electrode. Torresi et al. [195] polymerized aniline with PAAm hydrogel; they found that a positive current application could lead to the expansion of the PANI network and cause the release of aniline.

Despite various studies reporting successful fabrications of electric responsive hydrogels and some of their applications in tissue engineering, major limitations still remained to be tackled. For instance, most of the conductive hydrogel drug release depends on the ionic exchange between the drug particles and the electrolytic media, resulting in the oxidation of the loaded drugs, reducing its functionality. Also in some studies, it was found that only charged molecules could be released. Meanwhile, big volume drugs (even charged drugs) cannot be released by electric control easily. What is more, some of the studies show passive loss of loaded drug molecules due to the fast diffusion from

hydrogel matrix while others suffer from burst release once the electric field was applied, rendering poor control of the release kinetics. More importantly, there is no research fabricating multi-functional conductive hydrogel, with properties enhanced due to combination use of different materials, engineered into 3D conductive scaffolds. The designed scaffold boasts not only external support, but also the ability to be loaded with bioactive agents, acting as therapeutic molecule carrier to deliver the agent in site for tissue regenerative purpose in a controllable “on-off” manner.

2.4.4 Conductive Hydrogel Materials

Conductive polymers can be categorized into two kinds according to their structure and composition difference, namely the composite conductive polymer and the structural conductive polymer. Composite conductive polymers are polymers filled with various conductive substances in their polymeric matrixes with the intent to increase polymer conductivities. Their preparations involve the incorporation of conductive additives, such as carbon nanotubes and metal particles. Structural type conductive polymers usually utilize the ‘doping’ method to add conductive functional groups to the originally non-conductive polymers, gaining intrinsic high conductivities. After doping, the conductivities of these materials are largely improved, even up to the levels of conductive metals [196].

For the fabrication of conductive hydrogel, polypyrrole, poly (Ethylenedioxythiophene) (PEDOT), polyaniline, polythiophenes, graphene are some of the most extensively investigated materials [63]. Among them, graphene has drawn wide attention due to its excellent conductivity, mechanical property and biocompatibility [60, 190, 197-199]. Graphene is readily available and easy to obtain and process. As fillers, graphene can greatly improve the mechanical properties of conductive hydrogels. Furthermore, with simple functionalization of carboxylate, graphene could be easily degraded enzymatically into non-inflammatory degradation products [60], making it an

excellent material to be used *in vivo*. Graphene was firstly discovered by Geim group, which received Noble Prize in Physics with this discovery. Graphene is usually characterized according to the number of the carbon layers within a specific planar sheet or according to functionalization (such as single layer, bi-layer, multilayer graphene, graphene oxide (GO) and rGO etc.) [60, 200-202]. Single layer graphene is composed of a single layer of carbon atoms bonded together in a 2D planar structure. Due to its high reactive surface energy, suspension in solution is usually difficult, making its applications tricky. GO is formed by the high degree of oxidization of single layered graphene. There are several functional groups on the carbon atom sheet such as carboxylic acid, epoxide and hydroxyl groups. These functional groups make GO readily soluble in water resulting in a pH-dependent negative surface charge [203]. Other groups such as epoxide (-O-) and hydroxyl (-OH) are uncharged. They are usually located at the basal plane of the carbon atom sheet, causing weak interactions and only reactive with free radicals [204]. Figure 2-14 below shows the structural composition of graphene and GO. rGO is a reversed product of GO, which possesses exceptional physical and chemical properties fairly similar to graphene. The exfoliated GO is an ideal alternative for producing graphene, which can be fabricated in large quantities from inexpensive graphite powder. This fabrication method is relatively easy and, coupled with the cheap price of rGO, this method has gained popularity among researchers [202, 205, 206].

One crucial factor to be tackled for conductive hydrogel fabrication is to obtain water-soluble rGO and rGO dispersion. Fabricating rGO dispersion is immensely challenging due to the ultra-high surface area of the graphene platelets, resulting in strong van der Waals and π - π interactions between the surfaces of sp^2 structures [207], making it highly insoluble. Previously, researchers mainly utilize hydrazine hydrate treatment, hydrogen plasma treatment or thermal heating method (heating GO to around 2000 degree) to fabricate rGO. While these methods can indeed successfully reduce GO, various limitations remain. For instance, hydrazine hydrate reduction method may introduce harmful organic groups to the surface of the rGO; high temperature heating treatment may destroy the

graphene platelets due to the release of carbon dioxide, reducing the rGO conductivity; hydrogen plasma treatment may not be able to reduce GO completely and efficiently. More importantly, the rGO fabricated with these methods shows difficulties in dispersing in water or PEG hydrogel (almost non-dispersible), which largely limited its applications [189, 198, 201, 203, 204]. In order to fabricate highly concentrated rGO dispersion, researchers tried to modify the reduction method or the property of rGO to increase rGO solubility. Dang et al. [199] added N, N-dimethylformamide (DMF)/water solution to GO during the hydrazine reduction process. The solvation effect of DMF reduced the graphene sheet aggregation and increased its dispersion. Feng et al. [203] utilized similar method by adding poly (vinyl alcohol) (PVA) into GO during the hydrazine reduction process. The fabricated rGO-PVA composite showed increased dispersion. Moghaddam et al. [208] utilized the amphiphilic nature of the GO to cause self-assembly monolayer formation at the oil-water interface; the water was then vaporized and resulted in the dispersion of rGO in polymer. Lin et al. [202] utilized chiral liquid crystalline surfactant to non-covalently modify rGO (the polycyclic conjugated structure could dismiss the surface π - π interactions). Luo et al. [209] utilized chitosan oligosaccharide to chemically reduced rGO and found the modified rGO increased its dispersion in acidic solution.

However, despite the increased dispersion of the fabricated rGO, some drawbacks are also noted. For instance, the functionalization of rGO may induce conductivity reduction. Also, most of the dispersion methods only increase the rGO dispersion ability in a certain solvent by a small extent. The dispersion of rGO in PEG hydrogel or water still remains problematic. Besides, many of the dispersion methods may introduce harmful solvents or functional groups, rendering the applications of such rGO dispersions to tissue engineering impossible. Chen et al. [197] was one of the few groups that managed to fabricate biocompatible rGO successfully. They utilized L-lysine and sodium cholate to fabricate rGO. Their rGO showed superior dispersion in water via the aid of the sodium cholate as stabilizer, which reduced the surface energy of the rGO. More importantly, L-lysine and sodium

cholate are both biocompatible, the fabricated rGO is greatly appealing for tissue engineering applications.

Figure 2-15 Schematic of the difference of structure composition of graphene and graphene oxide.

2.5 Summary

There is a growing demand for 3D *in vitro* biological tissue scaffold models [5]. These models are utilized for various fundamental investigations in research. For instance, angiogenesis [3], tumor cell intra- and extravasations [9], neuronal growth, or the behavior of blood-brain barrier studies are some areas of such investigations [9]. Moreover, affordable and reproducible tissue scaffold models are extensively needed for drug screening purposes [1, 3]. Ideal attributes of scaffold models for various applications include scaffold materials of microstructures with strictly controlled stiffness or stiffness gradients, pore-sizes, chemistry and bio-conductivity [6]. From the above literature review chapters, the major challenges in tissue scaffold fabrication are: 1. The fabrication systems are either too expensive/time-consuming or cannot control the structure integrity. 2. The material utilized for the fabrication nowadays cannot meet the demands of regenerative tissue engineering purposes.

Fabrication Technique/ System	Resolution	Advantages	Disadvantages	Reference
2D stiffness creation/ transparency mask	Micro	Fast fabrication, 2D tissue environment study	Does not meet the requirement for 3D tissue engineering study.	[5,6]
Gas foaming/ electro-spinning/ phase separation etc.	Micro	Fast fabrication, enable 3D tissue scaffold manufacturing	System difficult to control the structure shape, integrity, complexity and resolution.	[3,4,8,10,12-16]
Stereo-lithography/ 3D bio-printing	Micro	High precision fabrication, able to print any desired structure	Low productivity, expensive fabrication system.	[33,47,76,103-106]
Interference lithography	Micro/Nano	High precision fabrication, high productivity	Require accurate positioning system, expensive laser sources. Can only produce certain periodicity.	[36,37, 40, 121, 123-125]
Two-photon polymerization/ direct laser writing	Micro/Nano	High precision fabrication, down to nanometer features	Low productivity, very expensive fabrication system	[72, 109-111]

Figure 2-16 Comparison of different hydrogel scaffold fabrication methods regarding their fabrication resolution, advantages and limitations.

Figure 2-16 above shows the comparison of the different tissue scaffold fabrication techniques with respect to structure resolution, advantages and disadvantages. For the two-dimensional and serial in patterning technique that was widely used previously [5, 8, 18, 19], microfluidic creation of stiffness and transparent masks patterning enable only 2D cell behavior studies. The fabricated scaffolds from this technique cannot be used for 3D tissue engineering study purposes. Some newly adopted 3D scaffold fabrication methods such as gas foaming, electro-spinning and phase separation enable fast 3D scaffold construction. However, these systems show limitations in controlling the size, shape, integrity and complexity of the scaffold [7, 8, 17, 89]. Although stereo-lithography system can precisely control both resolution and shape of the hydrogel structures, SL system is usually very

expensive and comes with extensively time-consuming manufacturing processes [109]. Two-photon polymerization can produce high precision structure with down to nanometer features resolution. However, the system requires very expensive laser sources and its low productivity has largely limited its application [66]. Therefore, these limitations all call for a new system to be developed for tissue scaffold construction. The new system should be able to control the integrity, complexity and resolution of the structure in a low-cost, fast, large area fabrication manner.

It has been demonstrated that mask-based IL technology provides a favorable platform for fast 3D microstructure fabrication [10, 119, 122, 124, 125, 138, 142, 210]. Mask-based IL/HL utilizes phase mask to record and preserve original structures; original 3D image could be reproduced upon one-step reconstruction light exposure [131, 133, 144]. Not only the fabrication time for IL/HL is much shorter as compared to 3D printing, accurate system positioning is also not strictly required; largely reducing system costs and promoting improved stability. It also has the potential to control the feature size in the lateral and vertical directions accurately [38, 134, 135, 211]. To date, few studies have reported the use of IL technology to fabricate hydrogel tissue scaffold. This prompted us to explore the potential applications of mask-based IL technology on tissue engineering.

Furthermore, amongst the diversity of hydrogel scaffolding system, PEGDA hydrogel remains a popular choice as the fabrication material due to its well-acknowledged properties (such as intrinsic hydrated nature, photo-patterning ability, tunable tissue-like surface stiffness, and excellent biocompatibility), making it a close biophysical analog of natural ECM [61]. However, providing structural support and adequate biocompatibility are no longer the only emphasis in the hydrogel scaffold design [20].

Bioelectrical phenomena is widely found in human tissues and cells, such as neural, cardiac, bone and cornea etc. [57, 67, 163, 164, 166, 169, 180, 185], and it plays an essential role in cell signaling, cellular growth guiding and neo-tissue deposition [164, 165, 171, 179]. What is more, most

of these tissues require the delivery and participation of growth factors, drugs, enzymes, antibodies, nucleic acids etc. at the local injury sites to initiate and promote the healing process [165, 169, 179]. Conventional PEGDA hydrogel scaffold do not support optimal cell adhesion, transit electrical signal or deliver drug/particles [212]. As a result, a large family of electric-dependent cells such as neuron cells and osteoblasts has been found to grow unfavorably in the PEGDA hydrogel scaffold [170, 175, 180, 213]. This has become a major challenge for PEGDA scaffold tissue engineering application. Given the inherently favorable properties of PEGDA, further modification in bio-functional property such as conductivity and drug delivery ability has become the new focus. This inspired us to explore the possibility of modifying the PEGDA hydrogel into an electric responsive ‘smart’ hydrogel with controllable drug delivery ability for future regenerative tissue engineering applications.

Chapter 3 Ultra-fast, One-Step Three-dimensional Printing of Hydrogel Scaffold

This chapter introduces the concept and design of the proposed 3D printing system, which is capable of printing hydrogel scaffold structure efficiently. The key working mechanism of the printer is firstly demonstrated followed by the introduction of the optical system setting-ups and components design. Subsequently, two-layer hydrogel woodpile structure is successfully printed in less than 3 seconds with the system. It is almost 100 times faster than the conventional 3D bio printer (RegenHU BioFactory, Switzerland) utilized on the market. In addition, different experiment factors affecting the structure morphology are explored in order to optimize the experiment parameters and improve structure integrity. The surface nano-mechanical property and bonding strength of the crosslinked hydrogel are tested which could be beneficial once the scaffold is incorporated with microfluidic device in the future. Further, *in vitro* cell toxicity test of the printed hydrogel scaffold was conducted to confirm its biocompatibility.

3.1 Materials and Experiments

3.1.1 Polymer Matrix Formulation

Poly (ethylene glycol) Diacrylate with average molecular weight 700, 2,2,6,6-Tetramethyl-1-piperidinyloxy, free radical, 2,2,6,6, -Tetramethylpiperidine 1-oxy, TEMPO and solvent N-[2-(benzoylamino)-6-benzothiazolyl]-Tricyclo NVP-231 were purchased from Sigma-Aldrich (St. Louis, Missouri, United States), photo initiator Irgacure 2959 and methacryloxyethyl thiocarbamoyl rhodamine B dye were purchased from Ciba (Basel, Switzerland). The chemicals were used once

received. The hydrogel solution was mixed followed the protocol reported by Chen et al. [17]. Firstly, 90 mg TEMPO powder was dissolved in 10 mL NVP. Then 90 mg of Irgacure 2959 was mixed with 2 mL PEGDA and 0.1 mL of TEMPO-containing NVP solution. The 2 mL final solution composition results with 4 wt% I2959 and 0.04 wt% TEMPO. The solution was maximum vortexed for 1 minute and then put on the hot plate (50 °C) for 5 minutes. Solutions with different concentration of I2959 and TEMPO were prepared with the same method. Further, 200nM rhodamine B was added to the final solution for confocal microscopic imaging.

3.1.2 System Mechanism and Design

The working mechanism of the printing system is shown in figure 3-1. Collimated plane wave light propagates along the optical axis of the customized planar DOEs. The DOEs controls the luminous distribution of the incident light by diffraction: the diffractive property of the microstructure built up on the cover glass perturbs the phase of the transmitted light and diffracts the light onto different planes within the microfluidic device. Further, the focused light initiates the hydrogel crosslinking within the specific small region.

Each DOE patterns and focuses light onto a particular axial plane in the polymer solution. The focal length of the DOE increases in sequence, and spatial structure was formed by sequentially exposing these DOEs. Specifically, once the patterning of the DOE1 ends (first layer was formed), the translating stage moved and repositioned the DOE2 with a higher focal length for exposure, the light focused at a higher plane, crosslinking and solidifying the hydrogel at a higher plane.

Figure 3-1 Schematic of working mechanism of the 3D printing system.

The printing system was combined with a self-assembled microscope imaging system and a structure printing system, it is shown in figure 3-2A. All the components of the system were purchased from Thorlab Co. (USA). For the imaging system, white light LED kit was utilized as the light source. Microscope objective Nikon N100X-PFO with N.A 0.75 and working distance of 0.51 mm was utilized for light focusing. CMOS camera DCC1645C (with SXGA resolution (1280×1024 *pixels*)) was used to receive the images. The power of the white light is controlled by a power modulator (T-Cube high power LED driver). It has 6 different markings to adjust the brightness.

Figure 3-2 A: Overview of the 3D printing system, left: imaging system; right: printing system. B: Top view of the printing system. C: side view of the printing system. D: Printing system optical pathway. E: Detailed top view of the experiment well and sample holder, 1: DOE mask holder; 2,3: vale plate; 4: DOE mask.

The printing system is composed of a UV LED chip (as light source), a convex lens with 18 mm focal length (for light collimation), an optical shutter, an aperture with diameter of 2.5 mm, a Fresnel lens array chip, a sample holder, a translating stage and an experiment microfluidic container. The whole system is designed keeping a low cost in mind, and no expensive light source or components were used. The Fresnel lens array chip is light, can be easily replicated from the pre-designed wafer mould. Figure 3-2A shows the overall view of the whole system. Figure 3-2B and figure 3-2C show the top view and side view of the printing system respectively.

Figure 3-2D shows the detailed optical pathway approach of the printing system. From the bottom to the top, the non-coherent UV light (emitted from UV LED chip) was collimated by a convex lens (focal length 18 mm, positioned above the LED chip). The UV light was roughly focused after the collimation. The beam diameter at the focal point of the convex lens was tested to be around 5 mm. Further, a shutter is placed at the focal point of the convex lens to control the UV exposure time

during the experiment. The shutter blocks the light once is on and it is operated according to the pre-experiment time settings. Subsequently, the collimated UV beam propagates through an aperture with 2.5mm diameter. The aperture further reduces the beam diameter to approximately the same size as the base of the experiment vale plate. The final collimated light beam transmits through the DOE mask to the hydrogel solution in the vale plate, crosslinking the hydrogel solution.

Different layer structure is accurately printed via the aid of the motorized linear translating stages (PTE-Z8). It carries the DOE masks, and positions different DOEs under the vale plate for exposure sequentially according to the experiment settings. Figure 3-2E shows the detailed top view of the experiment well and sample holder. The whole experiment process is accurately controlled and automatically conducted via customized Matlab program. The printing pattern and the printing time can be controlled through the functions such as ‘mask loading’ and ‘experiment time setting’ etc. The Matlab program interface is shown in the figure 3-3.

Figure 3-3 Customized Matlab program interface.

3.1.3 Design of Linear Binary Fresnel Lenses

Fresnel lens has wide applications in condenser systems, solar energy gathering and acoustic sound wave focusing. However, not many researches have utilized the Fresnel lens for hydrogel scaffold fabrication applications. In this work, we designed a series of binary Fresnel lenses/Fresnel zone plates; these lenses were used for controlling the luminous distribution of the incident light.

The working mechanism of Fresnel lens is simple: assuming the refracting phenomenon only occurs on the surface of the convex lens. If we retain the curvature of the lens surface while removing the rest of the optical material, we could obtain a thinner and lighter optical lens with similar focal length [214]. Figure 3-4 below shows the schematic of the working mechanism of Fresnel lens. Unlike conventional convex lens, which focuses light due to refraction, Fresnel lens gathers the incident light

source via diffraction. Figure 3-4A a shows the structures of a binary Fresnel lens/zone plate and figure 3-4A b shows the structures of a multi-level Fresnel lens.

Figure 3-4 Working mechanism of the Fresnel lens. A: Multi-level Fresnel lens utilizes step-like structure to better approximate the curvature of the aspheric lens. B: Schematic of optical path lengths difference of the diffracted beams creates a focus point by constructive interference.

With the advance of micro fabrication techniques, multi-level Fresnel lens was further introduced. Multi-level Fresnel lens utilizes multiple phase levels to mimic the phase curvature of the spherical focusing domain (shown in Figure 3-4A b). The optical path difference of the diffracted beams differs by an integration number of wavelengths, and the resultant constructive interference creates a focus point (Figure 3-4B) [215]. By changing the level size and level number, the minimal focal length of the lens and the phase of the incident light can all be controlled. The diffraction efficiency for such multi-level Fresnel lens can be written as [214]:

$$\eta = \left[\frac{\sin(\frac{\pi}{M})}{(\frac{\pi}{M})} \right]^2 \quad (3-1)$$

Where M is the number of the phase level, the diffraction efficiency of the lens increased with the number of phase level, at the cost of more complex manufacturing procedures. For instance, when the level number is 4, the efficiency is 81%; while when the level number is 16, the diffraction efficiency is 99%.

What is more, the multi-level Fresnel lens structure can be further simplified and converted to 2D binary structure, which is called Fresnel lens zone plate or binary Fresnel lens. The zone plate is composed of alternating transparent and opaque zones, with a distance from the central axis proportional to the square root of the integer multiples of the operating wavelength [215]. Every other opaque zone blocks the incident light while the transparent zone transmits the incident light [216]. Similar to the multi-level Fresnel lens, the zone plate is defined by the zones. The parameters of the zones can be obtained by calculating the optical path from the DOEs to the focal points. Which allows a maximum geometric path difference of $\frac{\lambda}{2n}$ (where λ represents the incident light wavelength, and n represents the refractive index of the lens). The multi-level structure can be simply converted to the zone plate by a duty ratio of height: $t_i = 1 - h(x_i)/h_{max}$ in each interval g [217]. In the interval, the width of the air is giving as gt_1 , showing in figure 3-5.

Binary Fresnel lens/Fresnel lens zone plate only requires a flat surface, making the structure mounting straightforward [215]. Thus, the relief structure could be easily produced with etching and lithography technique, increased the tolerance to manufacturing errors [215]. What is more, comparing with the spherical lens, the fabrication process of the near-planar structure does not require polishing and gridding procedure. Thus, in this research, in order to reduce the manufacturing cost while maintaining relative high diffraction efficiency, two-level ($N=2$) zone plate (converted from 4-level structure) with pattern of lines and spaces were designed and fabricated, shown in figure 3-5b.

Figure 3-5 Structure and mechanism of the binary Fresnel lens: a. The conversion of the multi-level structure to the binary zone plate by duty ratio of height $t_i = 1 - h(x_i)/h_{max}$; b. Schematic of structure conversion from 2 level structure and 4 level structure to binary Fresnel lens/zone plates.

The main parameters to be taken into consideration when we design binary Fresnel lenses are: the overall size of the lens L , the minimal focal length f , the refractive index of the lens n (1.5255 for cover slip), the refractive index of the hydrogel n_0 (1.33 for PEGDA hydrogel), the diffraction efficiency of such lens (mentioned earlier) and the wavelength of the incident radiation λ (UV light 365 nm).

The radial distance for each zone is given as [199]:

$$r_k = \left[\left(z + k \frac{\lambda_{cm}}{M} \right)^2 - z^2 \right]^{1/2} \quad (3-2)$$

Where, z is the focal length of the lens, M is the number of the phase levels. λ_{cm} represents the wavelength of the incident light.

The level-height difference h of the multi-level lens before conversion can be obtained from the equation below [214]:

$$h = \frac{1}{Nf \left(\frac{1}{v_{cm}} - \frac{1}{v_s} \right)} \quad (3-3)$$

Where, f is the frequency of the transmitted light, v_{cm} is the light velocity in the hydrogel; v_s is the light velocity in the lens; N is the constant number.

The minimum focal length of general Fresnel lens can be obtained from the equation below [214]:

$$\frac{\Delta}{\lambda} = \left(1 + \left(\frac{f}{r} \right)^2 \right)^{1/2} \quad (3-4)$$

Where Δ is the ratio of the period of the binary Fresnel lens, λ is the incident light wavelength; r is the distance from the optical axis, shown in figure 3-6. For binary Fresnel lens/zone plate, the minimal focal length f can be further written as [214]:

$$nf = n\sqrt{r_m^2 + (f - z_m)^2} + n_0 z_m \quad (3-5)$$

Where, (r_m, z_m) is the coordinate of the internal points of the linear structure corresponding to the zone labeled with index m , f represent the minimal focal length of the lens, n represents the refractive index of glass slip and n_0 represents the refractive index of hydrogel.

Figure 3-6 Schematic of the complete structure of the binary Fresnel lens/zone plate.

The resolution of the focused light spot is another primary design consideration. The resolution of the focused light represents the width of the focused beam w . The resolution of binary Fresnel lens can be written as [216]:

$$w = \frac{\lambda}{2NA} \quad (3-6)$$

Where, NA represents the numerical aperture of the system, λ represents the wavelength of the incident light. The numerical aperture can be further written as [43]:

$$NA = n \sin \theta = \frac{\lambda_0}{Ld_{min}} \quad (3-7)$$

Where n represents refractive index of propagation medium, λ_0 represents the free-space wavelength. Ld_{min} represents the minimal feature size of the lens. In this research, the NA was calculated approximate 0.55, which is highly competitive with that of macro-scale optics. Furthermore, the full width of the beam at half its maximum intensity after the light propagates through the binary Fresnel lens mask determines the axial feature resolution, and it can also be obtained from the equation below [214]:

$$W = k \left(\frac{df}{\lambda f_{min}} \right)^2 \quad (3-8)$$

Where d_{min} represents the minimum feature size of the binary Fresnel lens, λ is the incident light wavelength. k is constant parameter (for linear binary Fresnel lens is 3.1). Thus, we can conclude, the peak intensity and axial resolution both depend on the minimal feature size d , the binary Fresnel lens size D and focal length f .

Figure 3-7 Schematic of the full width of the beam at half its maximum intensity after the light propagates through the binary Fresnel lens.

The peak intensity after light propagates through the binary Fresnel lens mask is also another design consideration, as it may affect the PEGDA crosslinking efficiency. It can be obtained from the equation below [214, 217]:

$$\frac{I_{peak}}{I_{incident}} = \alpha \left(\frac{D f_{min}}{d f} \right)^\gamma \quad (3-9)$$

Where, I_{peak} is the peak intensity at the focal point, $I_{incident}$ represents the incident light intensity. D is the overall diameter of the binary Fresnel lens, for linear Fresnel lens, constant parameter α is 0.39, γ is 1, and d represents the minimum feature size of the binary Fresnel lens, f represents the focal length of the lens.

Based on the theory and calculation, a series of binary Fresnel lenses with different orientations and focal lengths were designed. The overall designed linear binary Fresnel lenses arrays

were organized with seven individual $2.5\text{ mm} \times 2.5\text{ mm}$ Fresnel lens (same size as the vale plate), in horizontal and transverse directions, which is shown in figure 3-7 below. The pitch between each individual line is 0.05 mm . The leftmost picture shows an enlarged image of the texture on a single line of the Fresnel lens. Starting from left to right, the focal point of the binary Fresnel lens arrays increased from 88 mm to 438 mm , with 50 mm intervals.

Figure 3-8 Schematic of designed linear binary Fresnel lens arrays. The size of each individual lens pattern is 2.5 mm square. The pitch between each beam pattern is designed to be $50\text{ }\mu\text{m}$, the focal length of the lens was designed to increase from $88\text{ }\mu\text{m}$ to $438\text{ }\mu\text{m}$, with $50\text{ }\mu\text{m}$ intervals.

3.1.4 Fabrication of Super-thin Binary Fresnel Lens

The mold of the binary Fresnel lens was fabricated on a silicon wafer by projection photolithography method [218, 219]. $125\text{-}\mu\text{m}$ -thick Si wafer was utilized as the substrate. Firstly, hexamethyldisilazane (HDMS) was evenly spin coated (Laurell Technology, WS-400) on the surface of the substrate to improve the adherence between the substrate and the SU-8 photo resist. Subsequently, the wafer surface was spin coated with SU-8 positive photo resist followed by pre-baking for 12 hours at 80

degree. The pre-designed diffractive patterns were then placed above the SU-8 photoresist for UV exposure. As a result, binary Fresnel lens features were obtained by the above procedure. The solidified pattern (binary Fresnel lenses pattern) was developed for 24 hours after the exposure, yielding the final mold (shown in figure 3-8).

Figure 3-9 Fabricated diffractive mask mold.

The silicon wafer with DOE patterns were utilized as ‘masters’ for fabricating the transparent binary Fresnel lens mask by nano-imprinting. Specifically, a thin film of high modulus PDMS (10 MPa, VDT-731, HMS-301, Gelest, Morris-Viile, PA) was evenly spin coated (POLOS450, Netherland) on the mold at 1000 rpm for 40 sec, followed by spinning the wafer at 500 rpm for 30 min in order to achieve uniform wetting and coating of the PDMS. Subsequently, the mold was put in an 80-degree oven for 1 hour for curing. The fully cured PDMS was carefully peeled off from the master mould to yield the binary Fresnel lens mask pattern. Further, the transformation of the pattern from the PDMS to the cover glass followed the same procedure. A thin film of UV curable resin was evenly coated on the glass slip. Then previously fabricated PDMS phase mask was carefully placed on top of the resin, followed with UV exposure for 10 minutes and 2 hours development, yielding the final transparent binary Fresnel lens mask. Figure 3-9A shows the procedures of the fabrication process for the transparent Fresnel lens arrays, figure 3-9B shows the final transparent binary Fresnel lens mask,

which will be mounted on the translating stage for exposure. Figure 3-10 shows the FESEM images of fabricated the Fresnel lens pattern. Figure 3-10a shows the original binary Fresnel lens pattern on the wafer, figure 3-10b shows the transformed binary Fresnel lens pattern on the cover slip. From the enlarged image (figure 3-10c) it can be found there are slightly disorientations of the transformed the pattern at some place of the lens. However, most of the pattern on the cover slip is highly defined and having width almost the same as that of designed wafer mould (confirmed with figure 3-10d and e)

Figure 3-10 A: Schematic of the fabrication process of the transparent linear binary lens with the aid of integrated circuit technology based on wafer mould. B: Fabricated super-thin transparent binary Fresnel lens.

Figure 3-11 FESEM images of fabricated the Fresnel lens pattern: a. Transverse binary lens pattern on wafer mould; b.

Fabricated transverse binary lens pattern on cover slip; c. High magnification image showing the detailed surface morphology of the fabricated transverse binary lens pattern on the cover slip; d. High magnification image of the fabricated transverse binary lens; e. High magnification image of the fabricated horizontal binary lens

3.1.5 Printing of 3D Hydrogel Woodpile Structure

Figure 3-10A below depicts the two-layer woodpile structure printing process. The polymer solution was injected in the customized experiment well plate ($2.5\text{ mm} \times 2.5\text{ mm} \times 2.5\text{ mm}$). Then the well plate was fixed on four rods, positioned approximately $100\text{ }\mu\text{m}$ above the transparent linear Fresnel lens arrays (DOE). Subsequently, the Fresnel lens arrays were mounted on the translating stages and carried/positioned by the stage. The intensity of the incident UV light was measured around 30

mW/cm². Each array of the DOE on the phase masks is exposed in turn then shifted in relation to the other substrates. The focal length of the Fresnel lens arrays increases in sequence and the spatial structure was formed by sequentially illuminating these Fresnel lens arrays. Specifically, once the illumination of the first Fresnel lens ends (a layer was formed), after waiting for a certain time (delay time), the translating stage moved and positioned the second Fresnel lens with higher focal length under the well plate for UV exposure, the light focused at a lower focusing plane, crosslinking and solidifying the hydrogel at a different plane. In the end, beam structures with horizontal and transverse orientations forming at different levels were obtained. Furthermore, we examined the effects of illumination time and chemical concentration on the printed structure morphology.

After the experiment, the remained uncross-linked chemical was removed from the well plate. Pure PEGDA was carefully injected repeatedly to wash away the residual chemical in order to terminate the crosslinking reaction. Micrographs of the woodpile structures were immediately taken with the self-assembled phase contrast microscope, followed by confocal microscope imaging (Alba FCS, Germany).

Post-experiment treatments were conducted to ensure the samples were dried for FESEM imaging. Specifically, the formed structures were carefully removed from the well plate onto a clean cover glass. Then, the samples were immersed in isopropanol solution for 1min. After development, the structures were dried in the 70-degree oven for 1 minute. Field emission scanning electron microscope (FESEM, JEOL 7600) with an accelerate voltage of 5 kV was utilized to examine the surface morphology of the structures.

Figure 3-12 A: Schematic of utilizing multiple 1D Fresnel lenses for manufacturing multiple-layer woodpile structures. B: Schematic of the fabrication process (cross-sectional) view.

3.1.6 Nano-indentation Test of Surface Nano-mechanical Property of Crosslinked Hydrogel

3.1.6.1 Hydrogel Thin Film Preparation

The PEGDA thin film was fabricated using two cover-slides and two 150 μm thick spacers, the method is shown as figure 3-11. Specifically, 150 μl chemical solutions were dropped in the center at the bottom of the cover slide. The spacers were placed on the left and right edge of the cover glass; afterwards, the top glass was slowly placed on the top of the spacer. Once in contact with the top cover slide, the droplet of chemical solution was trapped between the two cover slides due to the surface tension. Subsequently, UV light was utilized to crosslink the entrapped hydrogel. After crosslinking, top cover slip was carefully peeled off.

Pre-experiment surface treatments were conducted to change the properties of the surface of the cover slide to reduce the structure damage during peeling off process. Top cover slides were treated with Trichloro (1H, 2H, 2H-perfluoro-octyl) saline in order to reduce the bonding strength of the structure with glass slides [220]. Bottom cover glass was treated with 3-(Trimethoxysilyl) propyl methacrylate to enhance the bonding strength of the structure with glass slides.

Figure 3-13 Schematic of the procedure of solidifying thin hydrogel film.

After obtaining the crosslinked hydrogel thin films, nanoindentation tests were conducted to exam the effect of UV illumination time on the surface nano-mechanical property. In total 42 PEGDA samples with constant chemical concentration of 4.0 wt% I2959 and 0.04 wt% TEMPO were prepared (standard chemical composition). The samples were crosslinked under the UV illumination from 1.4 seconds to 4 seconds, with 0.2 seconds interval (light intensity 112.3 mW/cm^2). Six locations of each of the thin film were tested with high and low loads respectively in order to obtain an approximate accurate average result.

3.2.6.2 Nano-indentation

Specifically, the working mechanism of nano-indentation is shown as the figure below, the laser beam emitted from laser diode is initially focused on the back of the cantilever. It then reflects from the back of the cantilever to the photodiode spot position detector. During the scanning process, the cantilever will move with the change of the topography of the sample [106] (due to the interaction atomic force between the micro tip atoms and sample surface atoms). As a result, the reflected light beam will subsequently offset.

In the detecting process, the distance between the tip and sample remains in a few nanometers range. If the distance is too big, the information acquired will not be accurate. Meanwhile, if the distance is too small, potential damage may be done to the tip and the sample. Thus, the feedback system functions as the regulator: it regulates the voltage applied to the cantilever according to the change of the atomic force, and furthermore, changes the distance between the tip and the sample [106].

Figure 3-14 Schematic of working principle of the AFM testing.

In total, six phases are revealed from a standard force curve, which is shown in figure 3-13 below. First phase: the cantilever slowly approaches the sample, when the tip comes close to the sample surface, tip will suddenly jump to contact with the sample (due to the atomic attraction force). The second phase: the contact-operating regime (indentation). For this regime, a constant force is applied at the probe tip as it travels along the sample surface. At locations where there are differences in topographical features, the system changes the load applied to the tip to maintain constant contact of tip and sample surface. At the same time, the deflections caused by these changes will be registered by the laser and detector system. This deflection signal information will be transformed to Young's modulus. After the indentation, the probe will retract and slowly move away from the sample. During this phase, the adhesion phenomenon may happen between the tip and surface, resulting a negative deflection of the cantilever.

Overall, the most important data to be collected during experiment is the deflection of the cantilever during the indentation regime. These deflection data will be further analyzed to identify the surface nano-elasticity of the PEGDA thin films.

Figure 3-15 Schematic of the six phases of the nanoindentation process.

Figure 3-16 Schematic of the six locations tested for each of the hydrogel thin film.

3.1.7 Bonding Strength Test

In order to test the bonding strength of PEGDA and the cover slip, razor blade tests were conducted. Pre-experiment surface treatment was conducted to change the surface property of the cover slips. Specifically, cover slips were immersed in 2 v/v% 3-(trimethoxysilyl) propyl methacrylate (Sigma Aldrich, USA) in 95% ethanol for two minutes and rinsed with pure ethanol. The cover slips were then put in the 100°C oven for 30 mins to create methacrylate group on the surface of cover slips. After surface treatment, PEGDA thin films (150 μm thickness) were prepared by illuminating 5 minutes, 3 minutes and 1 minute using the same method stated in the previous section. Hydrogel solutions with varied chemical concentration were prepared using the same protocol stated before (I2959 concentration was changed from 0.5 wt% to 2 wt%). Subsequently, razor blade (IDL USA, 238 μm thickness) was manually inserted into the cover slip-PEGDA interface (in order to delaminate PEGDA from the cover slip) until the crack was stable; the experiment process is shown in figure 3-15. Afterwards, microscopy was utilized to record the figures of cracks and measure the average crack length.

Figure 3-17 Experiment set up for the razor blade testing [221].

The energy of delamination of the thin PEGDA film from the cover slip is associated with the crack length created during the demoulding process, and it can be calculated according to the equation below [221]:

$$G = \frac{3E_1 t_1^3 \delta^2}{(1 + \alpha \eta^3) L^4} \quad (3-10)$$

Where, E_1 is the plate modulus of the hydrogel, t_1 is the thickness of the crosslinked hydrogel; δ is a constant number, α is the ratio of the plate modulus of the polymer to that of the cover slip, η is the ratio of the thickness of the polymer to that of the cover slip, and L is the length of the crack.

3.1.8 Cell Toxicity Test

3.1.8.1 Cell Culture

Mouse conjunctival fibroblast cells were sub-cultured from wild type C57 mice conjunctival tissues. All experiments conducted with this cell type were within the first six passages. The cells were transferred to the 15 mL tube and then centrifuged at 300 xg at 37 degree for 5 mins, and then the supernatants were removed afterward and 3 mL fresh DMEM medium were added in the tube and well mixed. Subsequently, the cell was transferred to the T175 flask containing 20 mL of fresh DMEM medium. The flasks were then incubated under 37 degree and 5% CO₂ atmosphere for further use.

3.1.8.2 Cell Counting and Seeding

In our experiment, 5000 cells were seeded to each well for cell toxicity test. Specifically, 5 mL trypsin solution was added to the T175 flask, the flask was then transferred to the incubator for 5 mins in order to speed up the detachment of the cell from the flask. Subsequently, 10 mL of fresh DMEM medium was added into the flask (the flask was washed with the medium several times to make sure all the cells were detached). Then the medium with cells was transferred to 50 mL falcon tube and centrifuged for 5 mins with rotational speed of 300 XG in order to spin down the cells. After spinning down the cells, the supernatant was carefully removed and 2 mL of fresh DMEM medium was added into the cell afterwards. The solution was well mixed to ensure the cells were homogeneously distributed. 8 µL of cell suspension was aliquot into the haemocytometer and placed under the microscope to count viable live cells. Cells were then counted from the four large corner squares and the average number of cells was recorded and multiplied by 10 to get the total amount of cells present in 1 µL (the total amount of cells was calculated to be 250 per microliter). After the counting, the

solution was diluted with an appropriate amount of DMEM to achieve the desired cell concentration. Finally the 96-well plate was loaded to achieve approximate 5000 cells per well and the plate was then incubated overnight.

3.1.8.3 Toxicity Test of Printed Scaffold

The evaluation of the cytotoxicity of crosslinked PEGDA hydrogel was performed with the MTT cell proliferation assay. Specifically, 5 μ L trypsin solutions were added to the T175 flask, the flask was transferred to the incubator for 5 mins in order to speed up the detachment of the cell from the wall. Subsequently, 10 mL fresh DMEM medium was added in the flask (the flask was washed with the medium several times to make sure all the cells were detected). Then the medium with cells was transferred to 50 mL falcon tube and centrifuged for 5 mins with 300 XG rotational speeds in order to spin down the cells. After spinning down the cells, the medium was carefully sucked out and 2 mL fresh medium was added in the cell afterwards. The solution was well mixed to ensure the cells were homogeneously distributed. 8 μ L of the solution was collected and transferred to the hemocytometer to count the cell number (the cell number was calculated to be 250 / μ L). Then 18 mL fresh DMEM was added into the previous 2 mL solution. Finally 200- μ L of the solutions with approximate 5000 cells was seeded into the 96-well plates and then incubated overnight. After 24 hours, hydrogel scaffold was added to A1-A5 wells, B1-B5 was filled up with only cells for control experiment and C1-C5 was filled up with only DMEM medium in order to obtain the background absorbance value.

In total, 4 time points from 24 hours to 96 hours (four plates) were utilized to examine the toxicity of the hydrogel. For each time point, the cell culture medium and the hydrogel film was carefully removed from that plate, then 180 μ L medium without phenol red and 20 μ L MTT solution (5 mg / mL) were added to each well, followed by 4-hour incubation at 37 degree under a 5% CO₂ atmosphere. Finally the well plate was transferred to multimode micro-plate reader (Infinite M200,

Techan. Co, Switzerland) to measure the absorbance at 540 nm. Finally the relative cell proliferation (%) was calculated according to the formula below:

$$\text{Relative cell proliferation (\%)} = (\text{AB}_{(\text{sample})} - \text{AB}_{(\text{background})}) / (\text{AB}_{(\text{control})} - \text{AB}_{(\text{background})}) \quad (3-11)$$

Where $\text{AB}_{(\text{sample})}$ is the absorbance of the wells with hybrid hydrogel thin film, $\text{AB}_{(\text{control})}$ is the absorbance of the wells with cell only. $\text{AB}_{(\text{background})}$ is the absorbance of blank medium with MTT solution.

3.2 Results and Discussion

3.2.1 Fabrication of Two-layer Woodpile Structure

Figure 3-16 demonstrates a two-layer 3D woodpile structure of PEGDA with fully interconnected inner architectures printed using the system within only 3 seconds (top layer 1s, bottom layer 2s). Figure 3-16a shows the phase contrast microscope (focal length $z=138 \mu\text{m}$) image of the structure. It can be observed that after the UV illumination, horizontal and transverse beam structures solidified and formed within the hydrogel solution. The average size of the beam was measured around $38 \mu\text{m}$ and the spacing between neighboring beams was around $100 \mu\text{m}$. Both are approximately similar to the designed parameters. Figure 3-16b shows the confocal microscope image of the woodpile structure, the microscope focuses at the top layer ($138 \mu\text{m}$ above the cover glass). Some background auto-fluorescence could be observed around the formed structure, which was caused by the over crosslinking of the hydrogel due to the poor collimation of the light source, further confirmed with our FESEM images. Figure 3-16c and d demonstrate the structure morphology under FESEM. The images show that the formed interconnected structure: upper-layer beams sit above the bottom-layer beams.

The average height difference of the beams was measured to be 39 μm , indicating the 3D formation of the woodpile structure.

Also, partially crosslinked hydrogel was observed below or around the beam structures. Although unintended, these partially crosslinked regions could bind the entire woodpile structures robustly to the cover slips, securing the whole structures in position when introduced to cell cultures for *in vitro* experiments. Figure 3-16e shows highly waviness single beam structure, which can also be observed in some part of the two-layer structures, the shrinkage of the beam structure could be associated with undesired collimation of UV LED and light propagation pathway. Figure 3-16f shows the schematic of light propagation through the binary Fresnel lens. Besides the intended light focal point (which has the strongest light intensity, causing more polymerization of the chemical), there are regions exposed to stray weak UV light, resulting in some polymerization as well. In this case, the more polymerized structure may have bigger surface tension compared to the less polymerized region, causing the shrinkage. This hypothesis can be proved with the FESEM figures (figure C and D), whereby there are unintended crosslinked gel structure surrounding the two layered pattern, reducing the resolution of the grid. This drawback associated with the LED light source could be possibly reduced with a better-collimated light system. The finding suggests the limitation of the adopted method in this research, and that further characterization of the light source is required to optimize the system.

Figure 3-18 Printed two-layer woodpile structures, a: phase contrast microscope image of the 3D woodpile structure (with focal length $z=138\ \mu\text{m}$); b: confocal microscope image of the two-layered 3D woodpile structure (scanning focal length $z=138\ \mu\text{m}$); c: FESEM surface morphology of the 3D woodpile structure (angle view), average height difference of the two orientation beams measured to be $39\ \mu\text{m}$; d: FESEM surface morphology of the 3D woodpile structure (top view); e: phase contrast microscope image of the waviness single beam structure; f: simulated light path after the binary Fresnel lens and schematic of the light propagation induced unbalanced crosslinking behaviour along the z-axis.

3.2.2 Effect of Different Experiment Parameters on Structure Morphology

3.2.2.1 Effect of Illumination time

Figure 3-19 Phase contrast microscope image (focal length $z=138\ \mu\text{m}$) of the woodpile structure printed with different illumination time. The chemical concentration remained the same for the four experiments while the printing time changed (upper/lower), a: 0.2 s / 0.8 s; b: 0.6 s / 2.2 s; c: 0.7 s / 2.4 s; d: 1 s / 3 s; e: 1.5 s / 3.2 s. f: average beam width of the structures increase with the increase of irradiation time. The scale bar represents $100\ \mu\text{m}$.

Optical images (top view) in figure 3-17 show the morphology change of the two-layer woodpile structure printed with different illumination times. To maintain a fair test, the chemical compositions of the five samples were kept the same (3.5 wt% I2959 and 0.04 wt% TEMPO). Horizontally orientated beams (upper layer) were printed first followed by transversely orientated beams (lower layer). Figure 3-17a shows the beam structure with illumination time of 0.2 s / 1.2 s. Due to the

extreme short illumination time condition, the beam structures were not able to crosslink and form the structure. Figure 3-17b shows the structure with illumination time of 0.6 s / 2.2 s. The structure was evidently formed with the exposure condition and the average beam size for each layer was measured to be around 35 μm . However, due to the short illumination time (0.6 seconds) for printing the horizontal beams, the remained tensile and compressive residual stress within the beams was too strong to keep the structure integrity. It further reduced the adhesion force of the resultant structure on the substrate. Hence, the top layer beam structure was not stable and disorientations of the beams can be observed. However, the bottom layer beam structure still remained its integrity and stayed in paralleled position, and is not affected by the movement of the top layer; this phenomenon further proved the horizontal and vertical beam structures were formed in two different planes, which will not interfere with each other. Figure 3-17b shows the structure formed with increased illumination time of 0.7 s / 2.4 s. The structure formed with this condition was highly defined. The beam size for each layer was measured to be around 38 μm . The structure fabricated had great integrity, suggesting that the remained tensile and compressive residual stress within the beam structures was much lower than the previous case. The overall mechanical property of the structure was stronger as compared to that in figure 3-17a. This suggests that with longer crosslinking time, the width of the beam structure increases with dimensions closer to the designed Fresnel lenses parameters. Figure 3-17c shows the structure formed with a further increased illumination time of 1 s / 3 s. High degree of waviness of the horizontal beam was observed in the structure formed. The average beam size of both layers increased to 45 μm , much bigger as compared to previous conditions. Also, fragments of cross-linked hydrogel can be found around the beams. Figure 3-17d displays the structure with 1.5 s / 3.2 s exposure time. The size of the horizontal beam increased dramatically to 103 μm , the average size of the transverse beam also increased to 60 μm . A lot of cross-linked hydrogel and fragments of debris were found between each beam.

3.2.2.2 Effect of I2959 Concentration

Figure 3-20 Phase contrast microscope image (focal length $z=138\ \mu\text{m}$) of the woodpile structure printed with different I2959 concentrations. The printing time was the same for the four experiment but the I2959 concentrations varied, a: I2959 2.0 wt%; b: I2959 2.5.0 wt%; c: I2959 3.0 wt%; d: I2959 3.5 wt%; e: I2959 4.0 wt%; f: average beam width changed with different I2959 concentration under same experiment irradiation time. The scale bar represents $100\ \mu\text{m}$.

Optical images (top view) in figure 3-18 show the morphology differences of two-layer woodpile structures formed with varying I2959 concentrations. The illumination times for the two layers maintained the same at 0.7 s / 2.4 s. The printing process is same as stated before. Figure 3-18a shows the structure made with I2959 concentration set at low I2959 concentration 2.5 wt%. The upper layer horizontal beam structure was evidently formed with the experiment condition and the beam size was measured to be around $40\ \mu\text{m}$. Due to the low I2959 concentration, the amount of free radicals generated was not enough to form mesh structures. Thus, discontinuity of the beam structure was observed. Also, there is no clear trace of structure formation for the lower layer transverse beam.

Figure 3-18b shows the structure formed with increased I2959 concentration of 3.0 wt%. Beams were formed, however, the average beam size for both layer is $45\ \mu\text{m}$. The whole structure was not defined, some debris was found within the line structure, and discontinuity of the horizontal beam structure could still be observed, indicating incomplete crosslinking. Figure 3-18c shows the structure formed with higher I2959 concentration of 3.5 wt%. The structure formed was highly defined and the integrity of the structure remained, suggesting efficient crosslinking. The average beam size was $38\ \mu\text{m}$ and the pitch size was $100\ \mu\text{m}$, exactly the same as the designed parameter. Figure 3-18d displays the structure made with 4.0 wt% I2959. The resultant structure was defined. However, compared to the structure shown in figure 3-18c the average beam size increased to $53\ \mu\text{m}$, indicating a more extensive crosslinking execution. From the obtained results it can be concluded that the morphology of the formed structures depends on the chemical concentration of I2959. Specifically, if the concentration of the photo-initiator I2959 is too low, the free radicals generated upon illumination may not be enough to initiate the crosslinking process. Thus, no structure or blurred structure will be produced. Longer illumination time creates larger amounts of free radicals, enables more crosslinking of the hydrogel and results in more defined structures. However, once the free radical amount exceeds a certain threshold, the structure becomes messy and bigger than the designed parameter due to the over crosslinking behavior.

3.2.2.3 Effect of TEMPO Concentration

Optical images (top view) in figure 3-19 below show the morphology differences of the two-layer woodpile structures printed with five different TEMPO concentrations. The exposure times for the two layers were fixed and standardized at 0.8 s / 2.6 s and the I2959 concentrations were set at 3.5 wt%. The printing process is same as stated before, layer two (horizontal orientation) was firstly printed by exposure with the Fresnel lens 2 followed by layer one (vertical orientation) printed by exposure with the Fresnel lens 1. The delay time between printing the two layers was set at five minutes. Figure 3-

19a shows the structure formed with TEMPO concentration of 0.03 wt%. As we can observe, both of the beams formed were very uneven, as the width of the beam was not uniform, changed in different locations. Also, a large degree of waviness in the structure was observed, indicating that the mechanical property distribution within the structure was not homogeneous. The average widths of the two layer beams were measured much bigger comparing to the designed parameter, around 45 μm for the top layer and around 68 μm for the bottom layer. Figure 3-19b shows the structure formed with increased TEMPO concentration at 0.035 wt%. The structure formed was highly defined and the width of both beam structure was approximate the same as the designed parameter, 37 μm . Figure 3-19c shows a structure formed with a further increased TEMPO concentration at 0.04 wt%, one can find the defined structure started to grow bigger, as the average width of the top layer beam grow to 39 μm , while the average width of bottom layer beam grow to be around 52 μm . The integrity of the structure started to become weak, especially for the bottom layer, as we can see there are ‘scattered’ gelatins formed around the beams. Figure 3-19d shows a structure formed with a further increased TEMPO concentration at 0.045 wt%. Waviness of the structure was observed again and the average width of the beam structure especially for the bottom layer has become bigger, around 78 μm , and we can find the formation of the vertical beam tended to be difficult, and slightly crosslinked gelation was observed gathering around the vertical beam. Figure 3-19e displays the structure formed with 0.05 wt% TEMPO. Under this high TEMPO concentration, the structure formed was very loose and blurred; highly waviness can be found in the top horizontal beam. While the bottom vertical beam tended to ‘disappear’, showing no clear trace of formed outlines. Only a large amount of scattered gelation can be observed, making it impossible to measure the width.

Figure 3-21 Phase contrast microscope image (focal length $z=138\ \mu\text{m}$) of the woodpile structure printed with different TEMPO concentrations. The printing time was the same for the four experiment but the TEMPO concentrations varied, a: TEMPO 0.03 wt%; b: TEMPO 0.035 wt%; c: TEMPO 0.04 wt%; d: TEMPO 0.045 wt%; e: TEMPO 0.05 wt%; f: average beam width changed with different TEMPO concentration under same experiment irradiation time. The scale bar represents $100\ \mu\text{m}$.

3.2.2.4 Effect of Delay Time

Optical images (top view) in figure 3-20 show the morphology differences of the two-layer woodpile structures printed with five different delay time between printing each layer. The exposure times for the two layers were fixed and standardized at 1 s / 3 s, the I2959 concentrations were set at 4.0 wt%, TEMPO concentration was set to be 0.04 wt%. The printing process followed the same procedure stated before. Figure 3-20a shows the structure formed with no delay time (after printing the top layer, we directly printed the bottom layer). As it can be clearly observed, the average width of horizontal and vertical beams was way bigger than the primary design, $68\ \mu\text{m}$ and $98\ \mu\text{m}$ respectively. Also the

structure was printed with poor resolution and definition. Figure 3-20b shows the structure formed with increased delay time of 3 minutes. The width of the structure was smaller comparing to the previous condition, as the average beam width for the top layer was measured to be 60 μm , while 82 μm for the bottom layer. Figure 3-20c shows the structure formed with further increased delay time of 10 minutes. Waviness in the top layer beams was observed and the width of the horizontal beam structure was further reduced, measured to be 50 μm , average beam width of the bottom layer also tended to be smaller. Figure 3-20d displays the structure formed with 20 minutes delay time. We can find the structure formed was much more defined under this condition, the average width of the horizontal beam and vertical beams both reduced, measured to be 42 μm and 58 μm respectively. Figure 3-20e displays the structure formed with a further increased 30 minutes delay time. The average beam size for the two layers both reduced to be around 40 μm , approximately the same as primary design. The whole structure is defined and the feature resolution is much improved compared to the previous experiment conditions.

Figure 3-22 Phase contrast microscope image (focal length $z=138\ \mu\text{m}$) of the woodpile structure printed with different delay time between printing each layer. The printing time for each layer and the chemical concentration was kept the same, but the delay time between printing each layer varied during the experiments, a: 0 min; b: 3 mins; c: 10 mins; d: 20 mins; e: 30 mins; f: average beam width changed with different delay time between printing each layer. The scale bar represents $100\ \mu\text{m}$.

Thus, from the experiment results we can conclude, the four experiment parameter: illumination time, I2959 concentration, TEMPO concentration and the delay time between printing each layer all played significant roles in determining the final structures' resolution. In order to gain a deeper insight into the crosslinking phenomenon and to better address the morphology change of the structures formed with different condition, polymerization simulation was done. The results of simulated two-layer woodpile hydrogel polymerization process can be found in figure 3-21.

The approach here is to assume that during any given exposure step, the rate of polymerization is proportional to the square root of [intensity minus (α times the peak intensity in the lower focused line pattern)] [99], thus, the polymerization rate for each layer can be written as below:

$$R_{polymerization1} = \frac{1}{\sqrt{I_{pk1} - \alpha I_{pk1}}} \quad (3-12)$$

$$R_{polymerization2} = \frac{1}{\sqrt{I_{pk2} - \alpha I_{pk2}}} \quad (3-13)$$

Where, I_{pk1} represents the peak intensity at top layer, I_{pk2} represents the peak intensity at bottom layer; α is the inhibition rate as a proportion of peak intensity in bottom layer. Alpha varies from 0.5 to 10 percent in these simulations [222]. The exposure time for both layers is chosen such that the peak degree of polymerization anywhere in each pattern (if independently exposed) is 1 [223]. The degree of polymerization of the overall pattern is assumed to be the sum of the degrees of polymerization from the two exposure steps [224]. The gelation threshold is set to 0.3 times the peak degree of polymerization in either pattern [222]. The evaluate degree of polymerization after exposure for both levels can be written as below [222]:

$$Deg = R_1 \sqrt{Abs_{max1}^2 - \alpha I_{pk1}} + R_2 \sqrt{Abs_{max2}^2 - \alpha I_{pk2}} \quad (3-14)$$

Where, Abs_{max1} represents maximum absorbance of the light at top layer, Abs_{max2} represents the maximum absorbance of the light at the bottom layer. Therefore, it can be found that selecting TEMPO concentration, I2959 concentration, illumination intensity, exposure times are all critical for producing distinct patterns. Figure 3-21 below shows the schematic of the structure morphology of the two-layer woodpile structure regulated by different TEMPO concentrations. The concentration of the TEMPO reduces from images 1 to 6.

Overall, the different concentrations of each of these chemicals will result in different percentage of crosslinking, and therefore result in different structural texture. It can be concluded from the simulation results: 1. The morphology of the formed structures depends on four experiment factors, the chemical concentration of I2959 and TEMPO, the illumination time for each layer and the

delay time between the exposures of the two layers. Finding the best experimental conditions to produce the ideal defined structure with the designed parameters is the balancing process of these four factors. 2. Increasing the TEMPO concentration while maintaining the I2959 concentration will lead to delay of the structure formation. Increasing the I2959 concentration while maintaining the TEMPO concentration will accelerate the time of structure formation. 3. The ideal delaying time between two structures depends on the chemical concentration and illumination time.

Specifically, image f of Figure 3-21 below shows the simulation result of an ideal case of crosslinked two-layer woodpile scaffold with interconnection structures. The upper layer was firstly formed upon transverse DOE exposure. The interconnected structure in between the two layers maybe formed after the formation of the upper layer. The residual free radicals (generated after the first exposure) may sink and diffuse to the bottom layer during the delaying time, crosslinking the hydrogel along the vertical axis [222]. The completed final woodpile structure was generated after the exposure of the lower layer.

The concentration of the photo-initiator I2959 determines how much free radical can be generated at a single exposure. During a certain experiment reaction time, the more availability of free radicals, the more PEGDA will be cross-linked and the thicker the layer will be [225]. If the concentration of the photo- initiator I2959 is too low, the free radical generated upon illumination may not be enough to cause the crosslinking process. Thus, no structure or blurred structure will be produced. For example, the simulation results figure 3-21c and d show that the width of the formed beam structure is bigger than that of figure 3-21a. Also it can be found that the longer illumination time used (the larger the amount of free radicals will be generated) and the more defined the resultant structure will be. However, once the free radical amount exceeds a certain threshold, then the structure starts to become messy and bigger than the designed parameter.

TEMPO serves as the regulator of the crosslinking process. It attacks and reduces the effect of the free radicals [225]. After the exposure of the upper layer, the residual free radicals may diffuse to the lower layer. During this process, the radicals may bond with the PEGDA along the Z-axis forming the interconnection between the two layers. However, the probability of these interconnections forming is determined by the concentration of TEMPO. If the concentration of TEMPO exceeds the threshold of PEGDA crosslinking, then all the residual free radicals will be eliminated and no interconnections will be formed. If the concentration of TEMPO is lower than the required amount to eliminate the free radicals, there will be interconnection structures formed. For instance, simulation result figure 3-21d and c show the case when the concentration of TEMPO exceeds the threshold for the PEGDA to crosslink, and no interconnection structure is formed. Figure 3-21e and f show the case when the concentration of TEMPO is lower than the required amount, thus, the interconnection structure is formed. What is more, the diffused excessive free radical will eventually affect the ideal illumination time to create a defined lower layer structure. Therefore, the delay time between print two layers may be crucial, as it allows the quencher/TEMPO to bond with the over-produced free radicals to reduce or eliminate their effect on the fabrication of the first layer structure. This could possibly enhance the definition of the lower layer structure. However, how the free radical diffuses and what is the amount of the diffusion is not yet confirmed, there needs further study to better clarify the process and modify the system printing parameters.

Figure 3-23 Simulation of the two-layer woodpile structure formation process.

3.2.3 Nano-indentation Test

Figure 3-22 below shows the AFM testing results of the force-displacement curve of the hydrogel thin film prepared with different crosslinking time. Based on the displacement of the tip, we could calculate the surface nano-mechanical property of the thin film. Figure 3-23 below shows the nano-indentation test results of the crosslinked PEGDA thin film under different experiment times. In total 42 PEGDA samples with constant chemical concentration (3.5 wt% I2959, 0.035 wt% TEMPO) but varied experiment time (1.4 s to 4 s) were prepared. The experiments were conducted with AFM (JPK

instruments AG SM-02-0014) and a soft cantilever (PPP-ZEIHR, nominal force constant 20 N/m). During the experiment, two different kinds of loads (low, mid) were applied to the thin films. Different loads applied may affect the hydrogel response, under higher loads; the hydrogel exhibits higher stiffness value, while under low loads hydrogel shows a lower stiffness value. This is due to the in proportional increase in penetration of the tips travel in the hydrogel when we increase the applied load. Thus, a desired amount of indenting displacement on the hydrogel thin film is significant for obtaining accurate results. It was found during the experiment, when we applied low load (100 nN), the distance that tip travels into the hydrogel surface was less than 10% of the total thickness of the thin film, resulting high noise level indentation response. Meanwhile, when we use applied high load (1000 nN), the distance that tip travels into the hydrogel surface was more than 50% of the total thickness of the thin film, and the results shows extremely incorrect high value. As the tip penetrated into the thin film too much, the glass slip at the bottom may affect the elastic modulus reading. Thus, 500 nN load and 200 nN load were utilized in order to obtain reasonable tip penetration (20-30%). The figure 3-22 shows the elastic modulus values tested with six different locations on the thin film. It can be found that the elastic moduli of the six locations for every sample are almost the same, indicating the crosslinked PEGDA chain caused by UV illumination is evenly and homogeneously distributed.

Figure 3-24 AFM force displacement curve of the hydrogel thin films prepared under different experiment parameters a: 3.5 s under 500 nN applied load; b: 3.4 s under 200 nN applied load; c: Image of nano-indentation of the hydrogel thin film.

Overall, the UV irradiation time has a noticeable influence on the crosslinked PEGDA thin film surface nano-mechanical property. The surface nano-elastic modulus of the PEGDA thin film increase almost linearly with the increase of the illumination time. It shows the lowest elastic modulus with 1.4 seconds illumination time is only 0.3 and 0.5 MPa under high and low mode respectively. The highest elastic modulus with 4 seconds illumination is 3.4 and 3.9 MPa under high and low mode respectively. Such dramatic increase of the elastic modulus can be explained with the crosslinking density of the PEGDA polymer chain under varied crosslinking time. When the illumination time is small, the degree of the crosslinking density caused by photo-initiation is small, thus, it increased the degree of hydration in the PEGDA chain. Studies have identified that the degree of hydration dramatically affects the mechanical behavior of hydrogel [101]. The high degree water presence within the hydrogel network weakens the internal and external mechanical properties, resulting in a lower surface nano-elastic modulus [226]. Increasing the crosslinking time leads to more formation of irreversible covalent bonds, resulting in the increase of crosslinking density within the hydrogel

network. Meanwhile, researchers have found the elastic modulus of a given polymer increases proportionally to the increase of crosslinking density [100, 101]. The higher conversion rate cuts-off the distances between each molecule and increase the length of the polymerization chain formation of the hydrogel, and further, improves the overall elastic modulus of the hydrogel. Thus, it can be concluded by simply tuning the experiment time, we can print scaffold with varied surface nano-mechanical property for different tissue engineering applications.

Figure 3-25 Variation in the surface nano-elastic modulus of crosslinked PEGDA hydrogel thin films under different experiment time.

3.2.4 Bonding Strength Test

Hydrogel microstructures fabricated need to retain their integrity within the incorporated microfluidic system, both when uncrosslinked solution is flushed out after manufacture or during the use. Pressure gradients across the structure induce flow in patterned channels and permeation of the porous hydrogel, together with deformation of the gel structure and shear stresses at its boundaries; these stresses and strains may peel the hydrogel structure from the cover slip, leading to difficulty *in vitro* cell applications. Thus, the adhesion/bonding strength of the hydrogel structure to the cover slip or

surrounding device is crucial. The figure 3-23 below shows the micrograph of the formation of ‘cracks’ of the PEGDA after the demoulding. The bonding strength for each film type against the methacrylate treated cover slip was recorded as crack length shown in figure 3-24 below. The bonding strength of the hydrogel to the cover slip is directly related to the crack length caused by the inserted razor blade. The larger of the crack length indicates the smaller bonding strength in between the hydrogel and cover slip interface. As noted, the PEGDA thin film fabricated with 2 wt% I2959, 30 v/v% PEGDA and 5 minutes illumination shows the shortest crack length (around 1750 μm), indicating strongest bonding strength exhibits between the thin film and the cover slip. Meanwhile, the PEGDA thin film fabricated with 0.5 wt% PEGDA, 15 v/v% PEGDA and 1 minute illumination shows the longest crack length (around 2850 μm), indicating the weakest bonding strength between the thin film and the cover slip.

According to the data collected, the UV irradiation time has a noticeable influence on the crosslinked PEGDA thin film bonding energy. For instance, hydrogel thin film made with same I2959 concentration 2 wt% and PEGDA concentration 30 v/v%, the crack length for irradiation time 5 mins is measured around 1700 μm , for irradiation time 3 mins is measured around 1980 μm , while for irradiation time 1 min is measured around 2100 μm . The hydrogel thin film tends to show increased bonding strength with the increasing of illumination time.

Meanwhile, the I2959 concentration also shows to have an observable impact on the overall thin film bonding strength. For hydrogel thin film made with same I2959 concentration and irradiation time, the decrease of the PEGDA concentration leads to crack length growth after the insert of the blade. These phenomena can be associated with different crosslinking degrees of the PEGDA [62]. The increase of illumination time and I2959 concentration increases the crosslinking degree and density of hydrogel thin film, generating more bonded polymer chains. Meanwhile, as mentioned before, PEGDA thin film was fabricated on the cover slip treated with methyl methacrylate solution.

Therefore, the formed polymer chains will attach to the methacrylate functional groups on the cover slip, increasing both localized adhesive thickness and the contact area, leading to an increase in the bonding energy [227]. Similarly, the low degree of PEGDA crosslinking decreases the thin film overall crosslinking density, reducing the amount of PEGDA adhesive surface and contact area on the cover slip [221]. It further reduces the amount of PEGDA polymer chains bonded with methacrylate functional groups, causing the reduction in the amount of mechanical interlocking between the contact interfaces [228].

What is more, it can be found for hydrogel thin film made with same I2959 concentration and irradiation time, the increase of PEGDA concentration also increases the crack length. The demoulding of the PEGDA thin film from the cover slip is *via* the adhesive failure model. The demoulding process disbonds at the interface with all the adhesive PEGDA film on the cover slip completely separated, no visible residue adhesive PEGDA was left. Thus, when the film was prepared with higher PEGDA concentration, the increased viscoelastic property of the hydrogel resulted in a better wetting effect, enhanced the contact of the hydrogel with the surface of the substrate [228]. When the contact becomes close enough, it will be able to make molecular interactions (such as van der Waals force) with the cover slip. Meanwhile, the increased amount of PEGDA concentrations enhanced the hydrogen bonding chance with methacrylate groups, leading to increase of the bonding energy [221, 227]. Thus, the enhancement of the overall bonding energy for increased PEGD concentration is attributed to the improvement of hydrogel viscoelastic and the amount of bonded methacrylate groups to the polymer chains [226].

Figure 3-26 Micrograph of the formation of ‘cracks’ during the experiment of razor blade testing, the scale bar represents 300 μm .

Figure 3-27 Comparison of the crack length of the PEGDA with razor blade test under different experiment conditions.

3.2.5 *In Vitro* Toxicity Test

The results of the cytotoxicity experiment (figure 3-25) show the cell viability percentage of control and experiment groups. For every time point, the viability of the experimental cells was compared against the control group. For the experiment group treated with crosslinked PEGDA scaffold, no statistical difference was observed for the first 24 hours, as the cell viability percentage was around 93%. However, the percentage dropped to 63% after 48 hours. For the third time point, the experiment group cell viability percentage increased and reached 67%. Subsequently, by the end of the experiment, the experiment group cell viability percentage further increased to 74%. The cell toxicity test indicates, the crosslinked PEGDA hydrogel structure showed slight toxicity to the fibroblast cells over the concentration range examined, as there was around 40% reduction of the cell viability after the second time point, this may be caused by the relative high concentration photo-initiator Irgacure 2959 (I2959) utilized to crosslink the hydrogel, as previous research has demonstrated I2959 is slightly toxic to cells [5]. However, after the second time point, the cell showed signs of proliferation. This suggests that the photo imitator only affected the cells for a short period.

Figure 3-28 MTT cell proliferation assay test of the PEGDA hydrogel scaffold.

Chapter 4 Photo-patternable, ‘Smart’

Conductive Hydrogel for Controllable Particle

Release with One-Click Reaction

In the previous chapter we have examined the problems in current tissue scaffold fabrication techniques and have introduced the concept and design of the proposed ultra-fast 3D printer, which can overcome these problems. However the application of the 3D printing system is still largely limited by the usage of conventional PEGDA hydrogel. This is due to PEGDA hydrogel lacking the bio-conductivity and drug delivery ability, as explained in the literature review. This inspired us to explore the possibility of developing multifunctional hydrogel to fulfill the requirements. Hence, through this study, we investigated the synthesis and characterization of a state of the art photo-patternable ‘smart’ conductive hydrogel. This new generation of composite polymer is called ‘smart’ material as it could change their volume or release of substances once an external signal is applied. It not only preserves the well-acknowledged superiorities of the conventional hydrogels (such as hydrated nature, photo-patterning ability, tunable tissue-like surface stiffness, and excellent biocompatibility [59, 63, 64]), but also directly delivers through electric stimulations, the incorporated drugs to implanted cells realistic. The feasibility to utilize the ‘smart’ conductive hydrogel for controllable drug release application with click reaction is explored. In addition, hydrogel surface morphology change before and after electric treatment is systematically studied. Cell toxicity test is done to confirm the biocompatibility of the composite hydrogel. Furthermore, *in vitro* nano-particle viability study after the release is conducted to confirm the functionality of the released nano-particles.

4.1 Materials and Experiments

4.1.1 Synthesis and Characterization of High Concentration Photo-patternable rGO/PEGDA Conductive Hydrogel

The fabrication of high concentration, high yield colloidal rGO/PEGDA dispersion followed Chen group's work [197]. GO powder, l-Lysine (reduction agent), sodium cholate hydrate (graphene stabilizer), ammonia solution, PEGDA (Mn 700) and hydrazine monohydrate were directly purchased from Sigma-Aldrich, and the chemicals were used without any further modifications. 200 mg GO powder was firstly dispersed in 100 mL purified water and stirred for 1 hour. The dispersion was then centrifuged (SL 16 Centrifuge, Thermo Scientific, USA) for 20 minutes; the exfoliated residual GO was picked out. The evenly dispersed colloidal suspension of GO (2 mg/mL) in purified water was achieved by sonication with a high-power probe-type sonicator (Q700 Sonicator, Qsonica, USA) for 2 hours. Then 2 mL ammonia solution was added to the aqueous suspension of GO, the pH value of the solution was adjusted to around 12. Then 2.9 g l-Lysine and 2.2 g sodium cholate hydrate were added to the solution. The final solution was stirred for another 2 hours, and was put in autoclave (95 degree) for 48 hours afterward. The reduced rGO colloidal mixture was washed with purified water for three times and dried at room temperature for 24 hours. The final high concentration rGO and PEGDA dispersion was achieved by dispersing rGO with small amount of residual water in PEGDA hydrogel, then sonication with the probe-type sonicator for 1 hour. The schematic of the fabrication process is shown in figure 4-1.

Figure 4-1 Schematic of the fabrication process of high concentration rGO/PEGDA conductive hydrogel.

For comparison, normal hydrazine reducing method was also utilized to fabricate rGO also [229]. Firstly, GO powder was evenly dispersed in the water (2 mg/mL) by stirring for 20 minutes and then sonication with the same high power probe type sonicator for 2 hours. Subsequently, 2 μ L hydrazine monohydrate was added to the colloidal GO/water suspension, and it was stirred for another 30 minutes. The whole solution was put in the 95-degree autoclave for 48 hours for reduction. The obtained rGO colloidal mixture was washed with purified water for three times and dried at room temperature for 24 hours. The final rGO/water dispersion was achieved by dispersing rGO (with small amount of residual water) in PEGDA, then sonication with the probe-type sonicator for 1 hour. FTIR, UV-Vis and Raman spectrum were utilized to characterize the composite hydrogel.

4.1.2 Conductivity Testing of Composite Hydrogel

The conductivity tests were conducted to investigate the electrical property of the composite material. The experiments were carried out with self-assembled four-probe testing machine. Comparing to two-probe method, it does not have errors due to contact resistance of measuring leads. Specifically, crosslinked flat thin films specimens of the composite hydrogel (5 mm×5 mm×150 μm) were firstly prepared with the same method stated in the previous sections. Then the four probes (metal tips with finite radius) were attached to the sample in a straight line, the space S between each probe was set to be standard 1 mm. Afterwards, a high impedance current source was used to apply electrical current to the outer two probes, and a voltmeter was utilized to measure the voltage across the inner probe. Ten pieces of hydrogel thin films were used in each test. The resistivity is defined as:

$$\rho_0 = \left(\frac{V}{I}\right) 2\pi s \quad (4-1)$$

$$\rho = \frac{\rho_0}{G_7\left(\frac{w}{s}\right)} \quad (4-2)$$

Where, V is voltage across the inner probe, I stands for the current applied to the outer two probe, w is the height of the thin film, s is the space between each probe. The value of correction divisor function $G_7\left(\frac{w}{s}\right)$ can be found from the handbook. As in our case, the height of the film w is much smaller than the space between probes. Thus, the value of the correction divisor function approaches the case for an infinitely thin slice:

$$G_7\left(\frac{w}{s}\right) = \frac{2s}{w} \ln 2 \quad (4-3)$$

$$\rho = \frac{\pi w}{\ln 2} \left(\frac{V}{I} \right) = 4.5324 \times w \times \left(\frac{V}{I} \right) \quad (4-4)$$

Figure 4-2 Schematic of the four-probe method utilized for testing the conductivity of the hybrid hydrogel.

4.1.3 Fabrication of Layer-by-Layer Nano-particle

The synthesis of LbL poly Arginine-*siRNA* drug followed the procedure of our collaborator Tan group's previous work [230]. Positively charged hydroxyapatite particle (core) was firstly washed and dispersed in DI water, and then it was suspended in sodium chloride. The suspension (10 mg/ml) was added to 0.5 mg/ml negatively charged ARG solution, and vortex mixed for 10 minutes to coat the ARG layer. The ARG coated NPs were then washed with DI water again and suspended in sodium chloride. Afterward, 1.2 nmol of *siRNA* was injected in the solution to allow *siRNA* attach to the ARG layer. The final three-layer nano-particle was achieved by coating another layer of ARG to the outermost *siRNA* layer with the same procedure. Figure 4-3 below shows the fabrication process of three-layer nano-particle. Figure 4-4 below shows the structural composition of three-layer nano-particles.

Figure 4-3 Schematic of the LbL poly arginine particle fabrication process.

Figure 4-4 Composition of the layer-by-layer poly arginine-siRNA nano-particle. The nano-particle utilized negatively charged hydroxyapatite as core polymer, followed by attaching one positively charged layer of poly-L-arginine to the core, and then one negatively charged SPARC siRNA layer was attached to the arginine layer. The final LbL nano-particle was achieved by attaching another positively charged poly-L-arginine layer to the siRNA layer.

Preliminary test was done to calibrate the base fluorescence value of the fabricated poly arginine-FITC-*siRNA* nano-particle particle (also to confirm the fluorescence is available for detection by the micro-plate reader). Specifically, the schematic of the 96 well-plate setting up for fluorescence calibration testing is shown in figure 4-5. 200- μ l pure DMEM mediums were loaded in each well from A1 to A6. 1.4- μ l FITC tagged nano-particle was mixed with 200 μ l DMEM medium and then loaded in each well from B1 to B6. 0.75- μ l FITC tagged nano-particle was mixed with 200 μ l DMEM medium and loaded in each well from C1 to C6 for comparison. Wells from D1 to D 6 were left empty for control experiment; the plate was then transferred to the plate reader (Infinite M200, Techan. Co, Switzerland) to measure the fluorescence value.

Figure 4-5 Schematic of the 96 well-plate setting up for fluorescence calibration testing.

4.1.4 Nano-particle Release Testing

We designed and fabricated a simple microfluidic device to conduct the conductive hydrogel drug release test. Firstly, a through hole (5×5×5 mm) was cut on the poly (methacrylate) PMMA plate (15×15×5 mm) using laser cutting. The micro machined plates were cleaned by ethanol and DI water followed by ultra-sonication. Then the thoroughly cleaned plate was put under UV irradiation and

bonded to a cover glass with UV cured glue (Permabond, USA) for 5 min. Finally the Pt electrode was glued to the two sides of the inner walls of the well. Figure 4-6 below shows the schematic of the particle release device (cross-sectional view)

Figure 4-6 Schematic of the particle release device (cross-sectional view). LbL FITC tagged *siRNA* nano-particles encapsulated in the conductive hydrogel was injected in the experiment well and then crosslinked together. Afterwards, electric field was applied at the two side of the Pt-electrode, causing the release of the nano-particles from the hydrogel to the DMEM medium.

The trigger of nano-particle release study of the system was done with DMEM medium. The figure 4-7 (left) shows the overall set up for the release test; figure 4-7 (right) shows the detailed design of the microfluidic device. Control group (device without electric field treatment) and experiment group (device with electric field treatment) were done at the same time for comparison. The solution preparations for the experiments were stated as follow. For experiment group: firstly, 2 μ l poly-arginine *siRNA* nano-particles was maximum-vortex mixed with 100 μ l well dispersed PEGDA/rGO colloid gel (50/50 V/V %) for 10 min and then the solution was injected into the well,

followed by crosslinking under UV irradiation until fully crosslinked. The crosslinked gel was dried until stable mass was obtained, and then washed with DMEM medium in order to eliminate all possible soluble residues. Finally, 90 μ l DMEM medium was injected into the well (on top of the crosslinked gel). Varied potential (2 V- 4 V, with every 1 V interval) was applied to the end of two Pt electrodes for 2 hours to study the influence of the electrical field on the particle release of the system. At every time point (every 10 min), the DMEM medium was collected from the well and then injected with new DMEM medium. Finally, the collected DMEM medium was vortex mixed for 5 min and then loaded into 96 well plates and transferred to the multimode micro-plate reader (Infinite M200, Tecan. Co, Switzerland) to measure the concentration of free fluorescein in the solution. For control experiment group: same protocol was utilized to conduct the experiment but without electric field treatment. DMEM medium was also collected from the well every 10 min for two hours. Furthermore, for every set of experiment, 2 μ l poly-arginine siRNA drug particles was mixed with 90 μ l pure DMEM (triplicate) to obtain the reference base fluorescence value (to avoid the possible decay of the fluorescence and obtain a closer approximation of the release). The obtained release profiles were plotted as particle release percentage against release time point and cumulative particle release percentage against release time point.

Figure 4-7 Left: the overall experiment set up for the release test; Right: shows the detailed design of the drug release device.

4.1.5 Effect of Electric Current Flow on the Surface Morphology of Composite Hydrogel

In order to examine the effect of the electrical stimuli on the morphology change of the composite hydrogel, a series of experiments were designed and conducted. For control experiment 1 (pure PEGDA), the preparation of the pure PEGDA solution followed previous work, 10% (w/v) PEGDA in PBS was mixed with 4% (w/v) photo initiator I2959, the polymer solution was transferred to a cover glass and exposed to 365 nm UV illumination for crosslinking. For control experiment 2, previous fabricated rGO was firstly mixed with PBS solution (5 mg/mL), and then 50% (w/v) PEGDA and 4% (w/v) I2959 were added in the solution, the polymer solution was transferred to a cover glass irradiated for the same time for crosslinking. The crosslinked hydrogel samples were thoroughly washed in PBS and dried in the room temperature till stable weights were obtained. Then the samples were re-immersed in PBS solution and put in 37-degree incubator for 7 days until a constant equilibrium-swollen weight was obtained. Afterward, it was immediately frozen in -80-degree fridge for 24 hours and followed by freeze-dried for 48 hours. The final freeze-dried sample was cut in half

and sputtered with gold and put in SEM to exam the morphology. For control experiment 3, the preparation of the polymer solution followed experiment 2, after crosslinking, sample was thoroughly washed in PBS solution and dried in the room temperature till stable weight was obtained. Then Pt electrode was glued to the conductive hydrogel film and electrical voltage 2 V-20 V was applied to the electrode for 20 mins. After electric treatment, the sample was re-immersed in PBS in 37-degree incubator for 7 days and followed by the same procedure for freeze-dry. The final sample was cut in half and put in SEM to exam the morphology. The figure below shows the experiment set up for applying voltage to the crosslinked conductive hydrogel.

Figure 4-8 Experiment set up for applying electric field to conductive hydrogel.

4.1.6 *In Vitro* Cell Toxicity Testing

4.1.6.1 Cell Culture

Mouse conjunctival fibroblast cells were sub-cultured from wild type C57 mice conjunctival tissues. All experiments conducted with this cell type were within the first six passages. The cells were

transferred to the 15 mL tube and then centrifuged at 300 xg at 37 degree for 5 mins, and then the supernatants were removed afterward. 3 mL fresh DMEM medium were added in the tube and well mixed. Subsequently, the cell was transferred to T175 flask containing 20 mL of fresh DMEM medium. The flasks were then incubated under 37 degree and 5% CO₂ atmosphere for further use.

4.1.6.2 Cell Counting and Seeding

In our experiment, 3000 cells were seeded to each well for cell toxicity test. Specifically, 5 mL trypsin solution was added to the T175 flask, the flask was then transferred to the incubator for 5 mins in order to speed up the detachment of the cell from the flask. Subsequently, 10 mL of fresh DMEM medium was added into the flask (the flask was washed with the medium several times to make sure all the cells were detached). Then the medium with cells was transferred to 50 mL falcon tube and centrifuged for 5 mins with rotational speed of 300 XG in order to spin down the cells. After spinning down the cells, the supernatant was carefully removed and 2 mL of fresh DMEM medium was added into the cell afterwards. The solution was well mixed to ensure the cells were homogeneously distributed. 8 µL of cell suspension was aliquot into the haemocytometer and placed under the microscope to count viable live cells. Cells were then counted from the four large corner squares and the average number of cells was recorded and multiplied by 10 to get the total amount of cells present in 1 µL (the total amount of cells was calculated to be 250 per microliter). After the counting, the solution was diluted with an appropriate amount of DMEM to achieve the desired cell concentration. Finally the 96-well plate was loaded to achieve approximate 3000 cells per well and the plate was then incubated overnight.

4.1.6.3 Toxicity Test of Hybrid Hydrogel

The evaluation of the cytotoxicity of rGO-PEGDA hybrid hydrogel was performed with the MTT cell proliferation assay. After 24 hours of incubation, hybrid hydrogel thin films with different concentration were added into the 96-well plate and the figure below depicts the setup of 96-well plate for cell toxicity test. The hybrid hydrogel films samples were fabricated with different chemical concentration using the same method stated in the previous chapter. After fabrication, sterilized PBS was utilized to wash the samples for 1 min in order to eliminate all the possible residual uncrosslinked solutions before the samples were added to the 96-well plates. Crosslinked 75% rGO /25% PEGDA (v/v%) thin films were added to A1-A5 wells, crosslinked 50% rGO/ 50% PEGDA (v/v%) thin films were added to A6-A10 wells, crosslinked 25% rGO /75% PEGDA (v/v%) thin films were added to B1-B5 wells, crosslinked 75%rGO/25% PEGDA (v/v%) with 0.75 μ l nano-particle thin film were added to B6-B10, control cells were added to wells C1-C5 and DMEM medium mixed with MTT was added to wells C6-C10 in order to obtain the background absorbance value.

Figure 4-9 Set up of 96-well plate for cell toxicity test. A1-A5: 75% rGO /25% PEGDA (v/v%) thin film, A6-A10: 50% rGO/ 50% PEGDA (v/v%) thin film, B1-B5: 25% rGO /75% PEGDA (v/v%) thin film, B6-B10: 75%rGO/25% PEGDA (v/v%) with 0.75 μ l nano-particle thin film, C1-C5: Control group only cells, C6-C10: Control group only DMEM medium.

In total, 4 time points from 24 hours to 96 hours (four plates) were utilized to examine the toxicity of the hydrogel. For each time point, the cell culture medium and the hydrogel film was carefully removed from that plate, then 180 mL medium and 20 μ L MTT solution (5 mg / mL) were added to each well, followed by 4-hour incubation at 37 degree under a 5% CO₂ atmosphere. Finally the well plate was transferred to multimode micro-plate reader (Infinite M200, Techan. Co, Switzerland) to measure the absorbance at 540 nm. The relative cell proliferation (%) was calculated according to the formula below:

$$\text{Relative cell proliferation (\%)} = (\text{AB}_{(\text{sample})} - \text{AB}_{(\text{background})}) / (\text{AB}_{(\text{control})} - \text{AB}_{(\text{background})}) \quad (4-5)$$

Where AB_(sample) is the absorbance of the wells with hybrid hydrogel thin film, AB_(control) is the absorbance of the wells with cell only. AB_(background) is the absorbance of blank medium with MTT solution.

4.1.7 *In Vitro* Cell Testing of Released Nano-particle

4.1.7.2 Confocal Microscopy

Cells were carefully transferred to the four-well chambers (Lab-Tek, UK) using the same cell seeding protocol stated before (each well 500 cells, in total 6 wells, 3 wells for control group, 3 wells for experiment group) and then rested for 1 day. The release of the nano-particles from the conductive hydrogel followed the same procedure stated before. Three conductive hydrogel samples were encapsulated with *siRNA* nano-particles stained with fluorescein isothiocyanate (FITC). After crosslinking, 3 V electric voltage was applied to each of the 3 conductive hydrogel samples for 1 hour, and in total 300 μ L medium containing released nano-particles were collected (containing released *siRNA* nano-particles). Subsequently, 100 μ L-collected mediums were carefully loaded into each well. For control group, cells were not treated with anything. The four-well chambers were then incubated for 2 days in order to initiate cellular interaction of the released nano-particles with the cells.

Afterward, the cell-culturing medium was carefully removed from the wells and 500 μ L Hoechst[®] staining solution (diluting the Hoechst[®] stock solution 1:2000 in PBS) was added to the wells to cover the cells. The four-well chambers were incubated for 30 minutes covered with aluminum foil. Subsequently, the Hoechst[®] staining solutions were removed from the wells, 400 μ L 4 % paraformaldehyde (PFA) solution was added to each of the well and followed with incubation for another 15 minutes. Afterward the 4 % PFA solution was removed from the wells and 500 μ L PBS was added into wells and cells were ready for confocal imaging. All confocal images were taken with Olympus FluoView FV1000 (Japan, Tokyo) at 60X magnification and 2X zoom.

4.1.7.3 Protein Extraction

Cells were carefully transferred to the 12 well-plate using the same cell seeding protocol stated before (each well 5000 cells, in total 6 wells, 3 wells for control group, 3 wells for experiment group) and then rested for 1 day. The release of the nano-particles from the conductive hydrogel followed the same procedure stated before. Three conductive hydrogel samples were encapsulated with FITC tagged *siRNA* nano-particles; another three conductive hydrogel samples were encapsulated with scrambled *siRNA* nano-particles. After crosslinking, 3 V electric voltage was applied to each of the 6 conductive hydrogel samples for 1 hours, and in total 600 μ L medium containing released nano-particles were collected (3 sets of experiment group containing released *siRNA* nano-particles, 3 sets of control group containing released scrambled *siRNA* nano-particles) Subsequently, 100 μ L collected medium was carefully loaded into each well. The 12 well plates were incubated for 2 days in order to initiate cellular interaction of the released nano-particles with the cells. Subsequently, the supernatant was removed from the flasks, followed by washing the cells with 1 mL TBS solution. After removing the TBS solution, 150 μ L cell-lysis buffer (containing 1 mM β -mercaptoethanol, 1 $\times$ protease inhibitor and 1 $\times$ PhoSTOP) was added to each of the 6 wells to break up the cell membrane. Subsequently, the solution was maximum vortexed for 15 seconds and then followed by incubation on ice for 10

minutes. After incubation, the cells were spanned down (SL 16 Centrifuge, Thermo Scientific, USA) at 12000 rpm for 10 minutes at 4 degree. The cell lysate (containing extracted proteins) was then transferred to new tubes respectively and placed on ice for further use.

4.1.7.4 Protein Concentration Measurement and Sample Preparation

In order to confirm the protein concentration is enough for the western blot experiment, protein concentration measurement was conducted. Specifically, 1 μL of the cell lysate was withdrew from each of the six sample tube and added to 14 μL PBS (15 $\times$ dilution). 150 μL BSA standards (0 $\mu\text{g/mL}$, 2.5 $\mu\text{g/mL}$, 5 $\mu\text{g/mL}$, 10 $\mu\text{g/mL}$, 15 $\mu\text{g/mL}$ and 20 $\mu\text{g/mL}$) were prepared and added to each well of a 96-well flat bottom pate (duplicates for each standard). Then 5 μL of the diluted protein sample were added to 145 μL PBS solution to make a 450 times dilutions, the solution were vortexed for 1 min followed by centrifuging to spin down the proteins. Afterward, the diluted protein samples were added to the 96-well plate. Also, 150 μL Coomassie Blue was added to additional three wells to obtain a standard background value. Finally, the protein concentration was measured with the Tecan microplate reader at 595 nm wavelengths and the total quantity of the protein was calculated according to the measurement.

4.1.7.5 Gel Loading and Gel Electrophoresis Running

The gel loading plate and glass was throughly cleaned with 70% ethanal. Then the glass plate was placed into the gel frame and locked. Afterwards, MiliQ water was pumped into the gel to comfirm there is no leaking. Subsequently, equal amount (20 μL) of protein seperation gel was loaded into the wells of the SDS-PAGE gel, along with the marker, the seperating gel seperates protein in the sample based on their molecular weight, the procedure is shown in figure 4-10a. After gel loading, isopropanol was added into the gel tray in order to flatten out the gel, remove the air bubbles and block

oxygen. Well 1 was loaded with 7 μ L of ladder, well 2- well 4 were loaded with 40 μ g control group cell proteins (treated with scramble particles), well 5- well 7 were loaded with 40 μ g experiment group cell proteins (treated with SPARC loaded particles), well 8- well 10 were loaded with 40 μ L SB and water solution to obtain the background value. Subsequently, two electrodes were placed on the tank, followed with 90 V running for 30 minutes, then 120 V for 1.5 hours. Figure 4-10b-c shows the protein separation process during the gel electrophoresis running.

Once gel running procedure ends, the stacking gel was removed from the plate and the membrane was cut out as required. Then the membrane was placed in the Tupperware container and 100% methanol was poured on the membrane to permeabilize the PVDF membrane. Subsequently, the membrane was sandwiched using sandwich clamp following the order as: filter paper, PVDF membrane, gel, filter paper and sponge. Then the sandwich clamp was transferred into transfer tank and transfer buffer was poured into the tank (1 liter of transfer buffer is composed of 200 ml of 20% methanol, 100 ml 10 times Laemmli's and 700 ml MilliQ water). Subsequently, the gel was runned under 100 V in room temperature for 2 hours in order to transfer the gel membrane to PVDF membrane. After the transfer procedure, PVDF membrane was transferred to Biorad to measure the total transferred protein values and then PVDF membrane was ready for blocking.

Figure 4-10 Gel electrophoresis running process, the red arrow shows the protein separation process.

4.1.7.6 Sample Blocking, Antibody Staining and Film Developing

TBST buffer (1-liter TBST consists of 100 mL 10×TBST) and blocking buffer were prepared (consist of 0.1% final concentration of Tween 20 and 900 mL MiliQ water). 30 mL blocking buffer was made by dissolving 1.5 g skim milk powder in 30 mL 1×TBST. Subsequently, the membrane was retrieved in a plastic container and the proteins ladder band was marked with a marker. 10 mL of the blocking buffer was added to the container, and then it was placed in the shaking incubator (KS 4000 IC, UK) at 37 degree, 90 RPM for 30 minutes for blocking. After blocking, the blocking buffer was discarded, and then 10 mL primary antibody was added to the container followed by shaking incubation at 37

degree, 90 RPM for 30 mins for antibody staining. The membrane was rinsed with 1×TBST for three times (each time 10 minutes) after the staining. After rinsing, secondary antibody was added to the container and followed by shaking incubation at room temperature, 90 RPM for 1 hour. After secondary coating, the membrane was thoroughly washed with 1×TBST (under room temperature and shaking at 125 RPM) for 6 times (each time 5 mins). Afterward, the cleaned membrane was picked up from the container and placed on the Roche box, followed with adding 1.5 mL femto substrate solution (1:1 ratio) to the top of the membrane for the development. After developing for 3 minutes, the membrane was carefully transferred to the chemi-dot (Bio-Rad, USA) for imaging.

4.2 Results and Discussion

4.2.1 Synthesis of High Concentration Photo-patternable Conductive Hydrogel

Figure 4-11 below gives an overview of the comparison of fabricated rGO/PEGDA hydrogel (figure 4-11a,b), GO/PEGDA hydrogel (figure 4-11c), and pure PEGDA (figure 4-11d, the color change from GO (brown) to rGO (black) indicates the successful reduction of the GO). Figure 4-11a shows the fabricated high concentration rGO/PEGDA dispersion. It is observed that the dispersed rGO forms a homogeneous suspension in the PEGDA, and such stability was observed lasting at least for two month. This implies that the rGO can be readily dispersed in PEGDA hydrogel [196]. The excellent dispersion of rGO is attributed to the high-energy probe sonication treatment, which largely reduced the rGO particles size, increasing the overall surface tension. On top of that, graphene is stabilized by sodium cholate stabilizing molecules attached to the rGO sheets, modifying the rGO surface solubility, making it more hydrophilic [197]. Figure 4-11b shows rGO/PEGDA dispersion fabricated by conventional chemical reduction method. It was observed that the rGO flakes of differing sizes are not homogeneously distributed in PEGDA. This shows that the rGO particles in figure 4-11b cannot be fully blended into the PEGDA hydrogel, indicating after the reduction, the loss of the surface polarity increased the rGO hydrophobicity [231]. Figure 4-11c shows the GO powder dispersed in PEGDA hydrogel. The GO is homogeneously dispersed in the hydrogel. The good solubility is due to the existence of oxygen containing group in the rGO, which largely increases its surface energy [199]. The increase in the surface polarity also increases its hydrophilicity [206]. Overall, the excellent water-solubility and photo-patternable, printable ability of the composite hydrogel may facilitate its application in tissue engineering and scaffold fabrication.

Figure 4-11 Photographs of printable conductive hydrogel rejected from syringe pump (top) and colour change of different dispersion (bottom): (a) high concentration rGO in PEGDA (5 mg/mL), (b) rGO fabricated with hydrazine in PEGDA (1 mg/mL), (c) GO water aqueous solution, (d) pure PEGDA.

Figure 4-12 below shows the FESEM surface morphology comparison of GO and fabricated rGO. It can be observed that the GO demonstrates a thin flake-sheet like structure, which indicates the good exfoliation of graphite during the oxidation process. Conversely, the rGO shows much more wrinkled structures and corrugations, indicating that the rGO flakes were overlapped rather than aggregated [206]. The composite hydrogel was crosslinked under UV illumination once fabricated, and four-probe testing was utilized to measure its conductivity. The sheet resistance was measured to be $0.52 \text{ M}\Omega/\text{sq}$, and the thickness of the crosslinked composite hydrogel was measured to be $100 \text{ }\mu\text{m}$. Thus, the conductivity of such conductive hydrogel was calculated around $1.92 \times 10^{-4} \text{ S/cm}$.

Figure 4-12 FESEM images of the surface morphology of GO (A) and rGO (B).

4.2.2 Characterization of Photo-patternable Conductive rGO/PEGDA Hydrogel

The figure 4-13 below shows the Raman spectroscopy (Renishaw Invia, UK) results of the rGO, GO, PEGDA and rGO/PEGDA hybrid copolymer. The Raman spectroscopy provides the information of the crystal structure, disorder and defects in the graphene and PEGDA hydrogel. D and G bands are two of the most important features for characterizing graphene [232]. D band is associated with the structural defects or the disordered structure of sp^2 hybridized domain in graphene. The intensity of the D band is associated with the size of the sp^2 domain. The higher the D band intensity, the more sp^2 domains formed. G band is caused by the E_{2g} - vibration mode of the sp^2 carbon domain [232]. It appears as the stretching of the C-C bond in graphene and is used to compare the degree of the graphitization of the material. We can find from the Raman spectrum, before the reduction, GO has a D peak at 1350 cm^{-1} and a G peak at 1600 cm^{-1} . After reduction, the resulted rGO has a D peak at 1352 cm^{-1} and a G peak at 1598 cm^{-1} . This may be caused by the first order scattering of sp^2 C atoms [233]. The intensity ratio of the D band and G band is utilized to measure the disorder degree of graphene and the average size of the sp^2 clusters [234]. As it can be found from the figure, the G band is obviously down shifted after the reduction process, indicating the recovery of the hexagonal

network of the C-C bond with defect. The I_D/I_G value for GO is around 1.01, while the I_D/I_G value for rGO is around 1.1. This indicates after the reduction process, more graphitic domains are formed with increased sp^2 domains [235]. This further confirms that the GO is successfully reduced.

For the crosslinked PEGDA, vibration peaks at around 1500 cm^{-1} and 1730 cm^{-1} can be observed. The peak at 1500 cm^{-1} is due to the C=C bond of acrylate group in PEGDA, while the peak at 1730 cm^{-1} is attributed to the formation of carbonyl bonds caused by the chain crosslinking of acryloyl chloride and hydroxyl groups in PEGDA [225]. The peak at around 2800 cm^{-1} is caused by C-H stretching (CH_3) and C-H vibration (CH_2).

The spectroscopy of hybrid rGO/PEGDA hydrogel shows combined characteristics of both PEGDA and rGO. The D and G peaks, which cannot be found in PEGDA, appeared in the spectroscopy of the copolymer. The I_D/I_G ratio for the copolymer was observed as 1.1 (the same as rGO). Furthermore, the vibration peaks at 1500 cm^{-1} , 1730 cm^{-1} and 2800 cm^{-1} are also observed, implying the co-existence of crosslinked PEGDA and rGO in the polymer. This further confirms that rGO has been successfully incorporated in this copolymer.

Figure 4-13 Raman shift characterization of GO, rGO, PEGDA, and the hybrid hydrogel.

The FTIR-spectra (Shimadzu, UV-2450, Japan) of GO, rGO, PEGDA and PEGDA/rGO copolymer are shown in the figure below. Several different characteristic absorption bands of oxygen-containing groups can be observed from the GO spectrum. For instance, the absorption peaks around 870 cm^{-1} represents the aromatic C-H deformation. 1079 cm^{-1} represents C-O stretching, 1625 cm^{-1} represent the hydroxyl groups of molecular water (which is caused by the water used for diluting the rGO), 1729 cm^{-1} for C=O stretching [233]. A very broad peak at 3400 cm^{-1} is observed due to the O-H stretching vibration of C-OH groups.

Comparing to GO spectrum, the reduced GO spectrum exhibits different characters. For instance, the C-O stretching at 1079 cm^{-1} for rGO was largely reduced, indicating the successful removal of the oxygen bond from the GO structure [234]. Also, visible vibration reductions at 1728 cm^{-1} for C=O stretching and 3402 cm^{-1} for O-H stretching can be observed due to deoxygenation

during the reduction process. This indicates that the oxygen functional groups on the GO were removed and the GO was partially reduced [236].

The FTIR spectrum of crosslinked PEGDA exhibits characteristic bands at 2862 cm^{-1} , which represents the C-H bond, 1730 cm^{-1} , representing C=O stretching and 1115 cm^{-1} which represents the O-CH₂ bond. The FTIR spectra of PEGDA/rGO copolymers showed combined features for PEGDA and rGO. For instance, the intensity of C=O stretching at 1728 cm^{-1} was reduced as compared to PEGDA. Furthermore, there was a broadened band at 2862 cm^{-1} (C-H bond) in the copolymer, which is not present in rGO. The above findings confirm the presence of PEGDA as well as rGO in the copolymer.

Figure 4-14 FTIR spectra of GO, rGO, PEGDA and PEGDA/rGO copolymer.

The figure 4-15 below shows the UV-Vis spectrum (8001269-011-11, Agilent Tech, USA) results of GO, rGO, PEGDA hydrogel and hybrid hydrogel. It can be found that GO shows two

absorption peaks at 235 nm and 295 nm. The 295 nm shoulder represents the $n-\pi^*$ plasmon peak while the 235 nm shoulder represents the $\pi-\pi^*$ plasmon peak due to the transition of aromatic C=O bond. For rGO [207], a red shift could be observed as the spectrum shifted to 270 nm. This is due to the electronic configuration in the graphene sheet, as the C=O bond is embedded with reduced C-C ring, indicating the successful reduction of GO [234]. To obtain a deeper insight in the optical properties of the co-polymer, we compared the absorption spectrum of rGO/PEGDA with PEGDA. As observed from the figure, H aggregation at around 220 nm was observed in the copolymer, which was not observed for the pure PEGDA monomer. This confirms the successful incorporation of rGO in PEGDA monomer.

Figure 4-15 UV-Vis spectrum of A: rGO fabricated with 1-Lysine and B: original GO.

4.2.3 Characterization of Layer-by-Layer Poly Arginine-*siRNA*

4.2.3.1 FESEM Imaging

Figure 4-16 below shows the FESEM image of the fabricated *siRNA* loaded LbL poly-arginine nano-particles. From the image it was shown that average size of the nano-particles was around 80 nm in diameter. The nano-particles were relatively uniform in size. The nano-particles were observed to be spherical with relatively smooth surface morphologies.

Figure 4-16 FESEM image of the LbL poly-arginine nano-particles.

4.2.3.2 Fluorescence Calibration Testing

In order to quantify the nano-particles with the Tecan plate reader, LbL nano-particles were loaded with FITC-tagged *siRNA*. It was thus important to verify that the fluorescence intensity of the nano-particles changes proportionally with increased concentrations of fluorescence tagged nano-particles. Nano-particles of two different concentrations were dispersed in DMEM and loaded in a 96-welled plate. The samples were then tested with Tecan plate reader at gain 70. Figure 4-17 below shows the fluorescence values of the fabricated nano-particles. A1-A6 wells were filled with 200 μ l pure DMEM controls acting as the base fluorescence value. The average value of the six control samples was

around 401. 0.75 μl (0.03 mg HA, 0.072 nmol *siRNA*) poly arginine-FITC-*siRNA* drug particles dispersed in 200 μl DMEM were loaded in each well from B1-B6. The average fluorescence of the wells was around 693. 1.5 μl (0.06 mg HA, 0.144 nmol *siRNA*) poly arginine-*siRNA* drug particles dispersed in 200 μl DMEM were loaded in each well from C1-C6. The average fluorescence of the wells was 998. The fluorescence value of 0.75 μl poly arginine-*siRNA* drug particle could be obtained by using average fluorescence value of B1-B6 minus the average fluorescence value of the control DMEM, while the fluorescence value of 1.5 μl poly arginine-*siRNA* drug particle could be obtained by using average fluorescence value of C1-C6 minus the average fluorescence of the control DMEM.

Figure 4-17 Fluorescence value of the fabricated particle tested with plate reader system.

The mean fluorescence value of the drug particle is shown in figure 4-18 below. It can be found that the average fluorescence value of 0.75 μl poly arginine-*siRNA* drug particle was around 293 while that of the 1.5 μl poly arginine-*siRNA* drug particle was around 598. The results showed that the average fluorescence value of 1.5 μl poly arginine-*siRNA* drug particles was almost twice the amount of 0.75 μl drug particle. Thus it was conclusive that the fluorescence value increases with increasing concentration of FITC-tagged *siRNA* loaded nano-particles proportionally. This proves that the

particles loaded with FITC can be used to quantify the particle concentration with the Tecan plate reader.

Figure 4-18 Comparison of mean fluorescence intensity of the fabricated poly-arginine *siRNA* particles with 0.75 µL and 1.5 µL dosage.

4.2.4 Nano-particle Release Test

As mentioned in the previous chapter, Poly-Arginine FITC-tagged *siRNA* nano-particles were directly loaded into PEGDA/rGO hydrogel. Release study was then conducted on the hydrogels subjected to different voltage magnitude treatments in DMEM. The samples supernatant solutions were then analyzed for their fluorescence values using Tecan. At every time point, the medium solutions after release were collected and then completely replaced with fresh DMEM medium solutions to obtain a closer approximation of the release profile.

Figure 4-19 Fluorescence value of collected medium containing released particles (voltage applied 2 V).

Figure 4-19 above shows that the average base fluorescence value of pure DMEM medium was around 98. For the control experiment group (*siRNA* loaded nano-particles in DMEM subjected to no voltage treatment), the average fluorescence value over the experiment two hours period was detected to be around 95, indicating no obvious release of the drug particle when no electrical stimuli was applied to the hydrogel. E1-E12 shows the fluorescence value of the experiment group (particle loaded hydrogel subjected to 2 V voltage application over 2 hours). It was found that no release of the fluorescein was detected for the first two time points (E1-E2), and their fluorescence values were 97, 96 respectively (similar to control group). Observable release was found after 30 mins, with the fluorescence value of the collected supernatant increasing to 103. Subsequently, the fluorescence value increased almost linearly until the 60 minutes time point. From the 60 min time point till the end of the experiment, the release amount of the particle from the hydrogel stayed almost the same at every time point with fluorescence value of around 117, indicating relatively stable phase release.

Figure 4-20 below shows the initial average base fluorescence value of pure DMEM medium was around 90, while for the control group (*siRNA* loaded nano-particles in DMEM subjected to no voltage treatment), the average fluorescence value over the experiment two hours period was detected

to be around 89, indicating no obvious release of the nano-particles when no electrical stimuli was applied to the hydrogel. E1-E12 bars show the fluorescence value of the experiment group (particle loaded hydrogel subjected to 3 V voltage application over 2 hours). Observable release was found for the first 10 min after applying the voltage, as the fluorescence value increased to 102 directly. Subsequently, the fluorescence value increased stepwise and almost linearly and reached the peak value for 126 at the end of the experiment.

Figure 4-20 Fluorescence value of collected medium containing released particles (voltage applied 3 V).

Figure 4-21 below shows the average base fluorescence value of pure DMEM medium was around 120, while for the control group (*siRNA* loaded nano-particles in DMEM subjected to no voltage treatment), the average fluorescence value over the experiment two hours period was around 121, indicating no obvious release of the drug particle when no electrical stimuli was applied to the hydrogel. E1-E12 bars show the fluorescence value of experiment group (particle loaded hydrogel subjected to 4 V voltage application over 2 hours). Observable release was found for the first time point (10 min) after applying the voltage, as the fluorescence value increased to 129. Subsequently, the fluorescence value increased linearly and reached the stable value until the fourth time point (40

min). Then the value stayed around 145 from the 40 min to 110 min time points, indicating the release of the particle during this period was stable. Additionally, after the stable release phase, for experiment time point 110 and 120 min, a sharp increase of the fluorescence value (from 140 to 152) could be detected.

Figure 4-21 Fluorescence value of collected medium containing released particles (voltage applied 4 V).

Figure 4-22 below shows the average base fluorescence value of pure DMEM medium was around 283, while for the control group (*siRNA* loaded nano-particles in DMEM subjected to no voltage treatment), the average fluorescence over the experiment two hours period was around 281, indicating no obvious release of the nano-particles when no electrical stimuli was applied to the hydrogel. E1-E12 shows the fluorescence value of the experiment group (particle loaded hydrogel subjected to 20 V voltage application over 2 hours). Observable burst release of the particles from the conductive hydrogel can be found from the first time point (10 min) after applying the voltage, as the fluorescence value increased to 645. Subsequently, the fluorescence value decreased linearly. After the fifth time point (50 min), no release could be detected, as the fluorescence value was around the

same value as pure DMEM, indicating that the nano-particle loaded in the conductive hydrogel was possibly fully released from the hydrogel after 50 min.

Figure 4-22 Fluorescence value of collected medium containing released particles (voltage applied 20 V).

The figure below shows the drug released percentage profile with different applied voltage (figure A) and the cumulative drug release percentage profile (figure B) with different applied voltage. From the figures, for all sets of experiments, it was observed that the release percentage of the nano-particle almost increased linearly with experiment time. 7% of loaded nano-particles were released within 1 hour when the hydrogel was subjected to 2 V treatments, 18% for 3 V treatments and 29.7% for 4 V treatments. After 2 hours, 36% of loaded nano-particles were released when the hydrogel was subjected to 2 V treatments, 61% of particles were released for 3 V treatments and 81 % of particle were released for 4 V treatments. The results indicate that the voltage applied to the conductive hydrogel played a significant role in regulating the release behavior of the nano-particles from the conductive hydrogel. For every time point, the higher the voltage applied, the more particles were released into the DMEM medium.

Conventional hydrogel (as drug delivery carriers) release profiles usually exhibit initial burst release, making precise drug dosage release impossible. In comparison, from our release studies above, our designed hybrid hydrogel exhibits no leakage of particles (via diffusion from the hydrogel) and only when electric stimulus was applied, were drugs released with “click” reaction. Also, by varying the electric stimulus time and strength we could precisely control the dosage of the released drugs. This simple hybrid method may potentially enable more than one particle, macro-particles and hydrophobic particles included, to be loaded in the hydrogel.

Figure 4-23 A: *In vitro* particle release percentage profile from conductive hydrogel after electric treatments, applied voltage varies from 2 V-4 V. B: *In vitro* cumulative particle release profile from conductive hydrogel after electric treatments, applied voltage varies from 2 V-4 V.

4.2.5 Effect of Electric Current Flow on the Morphology of Composite Hydrogel

The mechanism of hydrogel drug release system relies on the unique physical properties of hydrogel: tunable porous structure which enables loading of drugs into the gel network, then subsequently release of the drug due to the molecule diffusion through the gel matrix. Some hydrogel with big pore sizes often results in drug diffuse/release with uncontrollable fast speed. Thus, the study of microstructure morphology and pore size changes of the composite hydrogel, before and after applying the electrical stimuli, could assist us in determining the right electrical parameters for conducting desirable drug release. By observing the morphology and pore size changes of the hybrid hydrogel upon different voltage treatments, we can potentially customize and control the right voltage treatments for different applications to achieve the desired release profiles of drugs.

In order to obtain a better insight of the particle delivery mechanism, FESEM imaging was utilized to observe the surface and cross-sectional morphologies of the crosslinked copolymer hydrogel with different treatment conditions. Figure 4-24A shows the overall and enlarged topography of crosslinked PEGDA hydrogel thin film. It was observed that the morphology of the crosslinked PEGDA is very smooth, no obvious pores or wrinkled structure was observed. Figure 4-24B shows the overall and high magnification topography of the crosslinked PEGDA/rGO hydrogel thin film. It can be found that the topography of such copolymer exhibited a completely different textured morphology; the super fine rGO particles (produced with high energy probe type sonication) were successfully blended into the hydrogel smoothly and homogeneously, increasing the overall surface morphology complexity. The high magnification images provide a better look into the unique surface texture of the copolymer. It shows that various wrinkled morphologies of different directions and sizes were formed on the surface of the copolymer. These wrinkles significantly enlarged the surface area of

the hydrogel, which can be beneficial for tissue engineering applications in multiple ways [237]. For instance, literatures have shown the surface wrinkles could significantly improve cell attachment and cell signaling even without any chemical modification [238-240]. Meanwhile, the increased surface area enables researchers to gain further control over the performance of drug delivery [238, 241]. In conclusion, such blended conductive hydrogel has great potential for tissue engineering and drug delivery applications.

Figure 4-24C shows the surface morphology of PEGDA/rGO hydrogel after applying electric voltage 2 V for 30 minutes. The high magnification image shows, after applying the electric stimuli, porous structures started to form on the surface of the hydrogel and the average pore size formed is around 5 μm in diameter. Figure 4-24D shows the surface morphology of PEGDA/rGO hydrogel after applying electric voltage 20 V for 30 minutes. It can be found a larger amount of porous structures were formed on the surface and the average pore size is around 17 μm in diameter, much bigger than the pores formed under the previous condition. Figure 4-25 below shows the average pore size and average pore number comparison of the rGO/PEGDA hydrogel surface before and after applying the electric voltage. It can be found that under certain electric treatment time, the average pore number and pore size formed on the surface of the hydrogel both increased with the increase of applied voltage.

Figure 4-24 FESEM figures of surface morphology of the crosslinked hydrogels. A: Pure PEGDA; B: PEGDA/rGO composite hydrogel without any treatment; C: PEGDA/rGO composite hydrogel after applying 2 V for 30 minutes; D: PEGDA/rGO composite hydrogel after applying 20 V for 30 minutes.

Figure 4-25 Average pore size and average pore number comparison of the composite hydrogel surface before and after applying the electric voltage.

Figure 4-26 below depicts the FESEM images of the cross-sectional morphology of the crosslinked PEGDA hydrogel, rGO/PEGDA composite hydrogel, and rGO/PEGDA composite hydrogel after applying voltage 20 V for 30 minutes. It can be clearly observed that the cross-sectional morphology of PEGDA hydrogel (figure 4-26A) is very smooth with no wrinkle or pores, while, the rGO/PEGDA composite hydrogel showed varied wrinkled structure (figure 4-26B), which is similar to the texture to the top surface. Figure 4-26C and 4-26D show the cross-sectional morphologies of the composite hydrogel after applying voltage 20 V for 30 minutes. High degradation of the hydrogel or hydrogel erosion can be clearly observed, the structure was interconnected with high-density porous structure. The images show that after applying electric voltage, the composite hydrogel suffers from high degree degradation.

Previously, Pillay et al. [47] reported electrical stimulus-assisted “ON-OFF” drug release method using polyaniline and poly (vinyl alcohol) based hydrogel. They observed electro-actuation (erosion of the hydrogel) phenomenon after electricity was applied to the hydrogel. Tsai et al. [187] also identified the erosion effect as the main control of drug release from polyvinyl alcohol/PANI hydrogel. The erosion of the hydrogel enhanced its permeability and promoted the diffusion of the

drugs. Similar to that, in our research, electrochemical reduction of the conductive hydrogel (erosion of the hydrogel) was also observed. During the mass transport process, the diffusion of the nano-particles from the hydrogel to the surroundings is the main mechanism causing the particle release [242-244]. Varied studies have proved that the diffusion process is involved in almost every step of the polymer transformation, such as polymer swelling, degradation and matrix erosion [242, 244-246]. For spherical models, the diffusion dynamic of the long polymer chains in the dense system follows de Gennes model [244]. The diffusion speed and dynamics are largely associated with the size, shape, aspect ratio, surface area, volume fraction of the nanoparticle, and its interaction with the polymer [244, 246, 247]. Previously, Maxwell et al. [248] developed a two-phase system to predict the diffusivity of the particle to the monomer. It was discovered that the large particles act as obstacles and result in decreased monomer diffusivity. In a recent simulation study by Karatrantos et al. [244], they discovered that reducing the size of the nano-particles could dramatically increase their diffusivity. They also found out that the interactions between the nanoparticles and the polymer chains appeared to hinder its chain motion; the stronger the interaction force, the lower diffusivity of the particles [243, 244]. Our experimental data also coincide with their simulation prediction. Given that the loaded nano-particles diameters are much smaller than the rGO/PEGDA entanglement strand. The nano-particles could diffuse in the polymer mesh much faster in comparison to the Stokes-Einstein predictions [249-251]. At the same time, the electrical field applied to the composite polymer induced the material surface morphology change. It weakened the interaction between the incorporated nano-particles with the polymer mesh, leading to a higher diffusion rate of the nano-particles.

Thus, from the release profile of the conductive hydrogel and the observation of surface morphology change after applying the electric field, it can be concluded that, electric treatments triggering nano-particle release from the hydrogel may be due to multiple effects. For instance, as the hydrogel degrades due to electrochemical reduction/oxidation, the permeability of the hydrogel changes, encouraging electric-field-driven induced charged nano-particles migration [46, 63, 187].

The observed hydrogel degradation phenomenon can be explained by electrostatic repulsion. Electrons were injected to the PEGDA chains when the polymer oxidized, causing mass transport from the polymer to the electrolyte. The resultant electrostatic repulsion leads to the expansion of the overall polymer network [187, 252]. Lira et al. [192] verified this with their polyaniline/polyacrylamide work, as they found the oxidation state change of the blended polymer could induce the volume change of the entire material, which lead to the drugs release from the polymer. Kim et al. [186] also reported such porous structure formation after they changed the charge state of their electrically responsive membrane PPy. Thus, it is possible to control the porous structure formation through the regulation of the polymer net charge. Furthermore, the overall polymer net charge change due to the electrochemical oxidation/reduction may further enhance the permeability of the hydrogel. Also, the positively charged outer layer of the nano-particle will be driven by the electric field and migrate towards the electrode bearing an opposite charge due to the electrophoresis and electro kinetic phenomena, resulting in the escape of the nano-particle from the hydrogel [187].

In conclusion, the release of nano-particle was not a result of a single effect. Only with combined actions of the above mentioned multi synergies can the nano-particle be released from the conductive hydrogel.

Figure 4-26 FESEM figures of cross-sectional morphology of the crosslinked hydrogels. A: Pure PEGDA; B: PEGDA/rGO composite hydrogel; C: PEGDA/rGO composite hydrogel after 20 V electric treatment for 30 mins; D: High magnification graph shows the high degree of hydrogel erosion

4.2.6 *In Vitro* Cell Toxicity Testing

Figure 4-27 below shows the micrograph of MTT assay cell toxicity testing. Figure 4-28 shows the results of the cell viability percentage of control and experiment groups' cytotoxicity experiment for the conductive hydrogel with different chemical concentration. For every time point, the viability of the experimental cells was compared against the control group. It can be found no statistical difference was observed for the experiment group treated with pure rGO chemical (yellow bar) throughout the four day time points as the cell viability percentage stayed around 96%, indicating the perfect biocompatibility of the fabricated rGO as conductive biomaterial fillers. For the experiment group treated with crosslinked PEGDA/rGO scaffold, no statistical difference was observed for the first 24 hours, as the cell viability percentage was around 93%. However, the percentage of 25% rGO, 75%PEGDA composite hydrogel and 50% rGO, 50% PEGDA composite hydrogel dropped to 62%

and 68% respectively after 48 hours. For the third time point, the 25% rGO, 75%PEGDA composite hydrogel group cell viability percentage increased and reached 65% while the 50% rGO, 50% PEGDA composite hydrogel viability percentage increased and reached 76%. Subsequently, by the end of the experiment, the experiment group cell viability percentage of 25% rGO, 75%PEGDA composite hydrogel and 50% rGO, 50% PEGDA composite hydrogel further increased to 74% and 84% respectively.

Overall, the cell toxicity test indicates, using the l-Lysine to reduce the GO is a relative biocompatible way to synthesis the conductive biomaterial fillers comparing to traditional chemical reduction method, the fabricated rGO showed non-toxic effect to the fibroblast cells over the time points and concentration range tested. The crosslinked PEGDA/rGO composite hydrogel structure showed slight toxicity to the fibroblast cells over the concentration range examined, as there was around 40% reduction of the cell viability after the second time point for both of the composite hydrogel (with different chemical concentration). As PEGDA has proved to be perfectly biocompatible to varied cell lines, this toxic effect may be caused by the relative high concentration photo-initiator Irgacure 2959 (I2959) utilized to crosslink the hydrogel, as previous research has demonstrated I2959 is slightly toxic to cells [5]. However, after the second time point, the cell showed signs of proliferation. This suggests that the photo initiator only affected the cells for a short period.

Figure 4-27 Microscope figure demonstration of *in vitro* MTT assay cell toxicity testing.

Figure 4-28 MTT assay proliferation test of the conductive hydrogel with varied concentrations.

4.2.7 *In Vitro* Cell Testing of Released Nano-particle

4.2.7.1 Uptake Study and Confocal Microscopy

The fabricated charged particles were attracted to cell membranes due to electrostatic interactions (as the surfaces of the nanoparticles were positively charged while the cell membranes were negatively charged). The attached particles were then up taken into the cells into the cytoplasm. Once inside the cytoplasm, the particles released *siRNA*, which then silence the intended RNAs, preventing the production of the target proteins. To confirm that the nano-particles were up taken into the cells successfully, confocal microscopy was employed to observe FITC-tagged nano-particles within primary C57 mice conjunctiva fibroblast cells. To confirm that the released particles retained their integrities and functionalities, western blot experiments were conducted to determine if they could still

knock down the intended proteins *in vitro*. Figure 4-29 below shows the confocal laser scanning microscopy images of the cellular interaction of released nano-particle after two days of incubation to cells. Figure 4-29 (a-e) shows the experiment group cells treated with the released nano-particle containing *siRNA*. Figure 4-29 (f-j) shows the control group cells without any treatment. The cell nuclei were stained with DAPI and the nano-particles were loaded with FITC tagged *siRNA*. It was observed from the phase contrast (a, f) and overlay pictures (e, k) that most of the released nano-particles were attached to the cell membranes with some successfully entering the cytoplasm of the cells. From the images, it was evident to infer that the particles retained their positively charged outer poly-l- arginine layer to be attaching onto negatively charged cellular membranes. Thus illustrating that the particle structural integrities were not compromised by the electrical treatments. These results correlate with the findings of Tan et al. [230].

Figure 4-29 Confocal laser scanning microscopy images (a-j) of cellular interaction of released particles; the scale bar represents 100 μm .

4.2.7.2 *In Vitro* Nano-particle Activity Study

It was proven that the released particles retained their structural integrities upon electrical treatments with the confocal imaging showing successful cell uptake. However, it was not verified if the sensitive therapeutic agent (*siRNA*) loaded within the particles retained its activity after the electrical treatments. To verify the agent activity, the released particles were treated to cells to investigate their effectiveness in down-regulating the intended protein (SPARC protein).

To test the proteins knock down effect of the released particle in cells, six sets of experiments were conducted. Experiment group cells were treated with released particles loaded with SPARC *siRNA*, while control group cells were treated with released particles loaded with scrambled *siRNA*. Both of the particles share the same spherical layer-by-layer structure. Figure 4-30 (1-3) showed the total spectrum of proteins from the lysed cells of control group samples (cells treated with released scrambled *siRNA* loaded particles) after gel electrophoresis. Figure 4-30 (4-6) was the total spectrum of proteins from the lysed cells of experiment group samples (cells treated with released SPARC *siRNA* loaded particles) after gel electrophoresis. It was found that all the protein separated in the stain free gels were successfully transferred onto the PVDF membrane with the Trans blot system. The total protein value amount was measured using Bio-Rad Chemidoc imager. The results after western blot assay were normalized against the total protein values of the respective samples. After normalizing, the samples can be compared to one another.

Figure 4-30 Bio-Rad chemidoc image of the total spectrum of proteins from the lysed cells: group 1 to 3 represents control group proteins (cells treated with released scramble particles), group 4 to 6 represents experiment group proteins (cells treated with released *siRNA* particles).

The normalization of band intensity against total protein for control group and experiment group samples was performed with Chemidoc Image LabTM software and the results were reported as the comparison for the total SPARC protein amount for the two groups [230], shown in figure 4-31. The analysis showed that the amount of SPARC protein detected from lysates of the cells treated with released scrambled *siRNA* loaded nano-particles was 40 % more than that of the cells treated with released SPARC-*siRNA* loaded particles, which confirms the protein knock down effect caused by the released nano-particles. The results showed that the SPARC-*siRNA* from the released nano-particle were still able to down regulate SPARC protein in cells by 40%. The result corresponded to the previous research done by Tan et al. [230]. They identified the SPARC-*siRNA* delivered by their LbL nano-particles could specifically reduce the target SPARC protein expression in fibroblast cells. Hence, the western blot results further confirm the nano-particles released from the conductive hydrogel after electric treatment retained their functionality. The efficiency of sensitive *siRNA* was

also not effected, indicating such conductive hydrogel could safely deliver sensitive agents in a controllable manner without compromising their activities. In conclusion, such photo-patternable multifunctional hydrogel can not only be fabricated to be conductive hydrogel scaffold, but also has the capability to be used as a therapeutic agent carrier, providing a safe platform to deliver required agents locally for tissue regeneration purposes.

Figure 4-31 Normalization of band intensity against total protein for control group and experiment group performed with Chemidoc Image Lab™ software. The sparac protein detected in control group cells was 40 % higher than that of experiment group cells.

Chapter 5 Conclusion and Future Work

5.1 Conclusion

In these studies, we developed a new 3D printing system for fast, one-step printing of 3D hydrogel scaffold. This system borrowed the idea of holographic lithography, combined with the joint usage of UV Led and binary Fresnel lens to accomplish low-cost, fast, automatic and simple 3D printing. Two-layered woodpile structures were successfully printed using the customized binary Fresnel lenses within 3 seconds. The results of phase contrast microscope and FESEM images demonstrated the integrity of the structure and proved the capability of the printing process. By varying the diffractive lenses, the system has the potential to flexibly pattern any designed structure within few seconds in the future.

Interestingly, it was also observed that there were uncommitted gel crosslinking surrounding the printed two-layer structure, reducing the structure integrity. This phenomenon can be largely associated with the poor collimation of the plane wave light source. The finding suggests that the limitation of the adopted method in this research, and further characterization of the light source is required to optimize the system. It also can be found the printing time and the chemical concentration determines the quality of the final printed structures. Finding the best experimental conditions to produce the ideal defined structure with the designed parameters is the balancing process of these factors. Therefore, further characterization of the experimental parameters of the printing process is required to verify the crosslinking mechanism of the hydrogel. This future work may potentially provide valuable information on crosslinking efficiency of the PEGDA chain, and it could eventually help to improve the existing DOE design and printing protocols.

The nano indentation test indicates the hydrogel surface nano-mechanical property increase with the increase of the UV irradiation time, which can be further related to the crosslinking density within the hydrogel network. As the longer UV irradiation cause a higher degree of chain crosslinking. Bonding strength test identifies the hydrogel thin film shows increased bonding strength with the increasing of illumination time, I2959 concentration and monomer concentration. The increase of these parameters improves the crosslinking degree and density of hydrogel thin film, generating more polymer chains, leading to larger localized adhesive thickness and the contact area, which resulted in increased bonding energy. The findings of the mechanical and the bonding strength test would serve as a good exploring reference in building foundations for translating the scaffold into practical applications. Cell toxicity tests indicated the crosslinked PEGDA scaffold showed slight temporal toxicity to the fibroblasts cells over the concentration range examined, due to the photo initiator I2959 utilized in crosslinking the hydrogel. However, the negative effect only lasted for a short period. Depending on application, we believed that this 3D printing system could be further improved and customized to print a wide range of scaffolds.

Additionally, in another research centred on developing functional hydrogel, we incorporated the water-soluble rGO with PEGDA and created a state of the art conductive hydrogel- ‘smart’ biomaterial which is electric-stimuli responsive. The photopatternable conductive hydrogel could be simply achieved by mixing the PEGDA with water-soluble rGO dispersion. Comparing to traditional chemical graphene oxide reduction method, the improved rGO dispersion fabrication method utilized biocompatible L-Lysine as the reduction agent and sodium cholate hydrate as the graphene stabilizer. The resultant rGO not only remained its high conductivity but also showed great improvement in the water/hydrogel solubility. FESEM figure, FTIR test, Raman spectrum and UV-vis graph analysis proved the successful fabrication of the conductive hydrogel. The feasibility to utilize the ‘smart’ conductive hydrogel for controllable nano-particle release application with click reaction was then quantified with a customized microfluidic device. Layer-by-layer nano-particles containing FITC

tagged *siRNA* drug were encapsulated in the conductive hydrogel then crosslinked together. Afterward, varied electric pulse (2 V-4 V) was applied to the hydrogel. The tested particle release profiles confirmed its controllable release ability. During the release experiment, the control group hydrogel (conductive hydrogel without electric treatments) showed no sign of releasing of the particles. While, for the experiment group (conductive hydrogel with electric treatments), the nano-particles encapsulated in the conductive hydrogel were tested released to the DMEM medium. During a certain experiment time, the higher the electric pulse applied to the hydrogel, the more nano-particles tested to be released. In addition, the hydrogel surface morphology change before and after applying electrical stimuli is systematically studied with FESEM. The FESEM results indicate the electric pulse may fasten the degradation rate of hydrogel. Porous structures were observed to form on the hydrogel surface after the electric treatments. The higher the voltage applied, the more and bigger the porous structure appeared, leading to nano-particles escaping from the hydrogel. However, the electric field caused hydrogel erosion may reduce the future scaffold mechanical integrity and mechanical property, resulting in scaffold failure before damaged tissue's healing. Therefore, further characterization of the experimental parameters of the electric parameter is required to verify the sustainability of the hydrogel for *in vivo* cases.

Cell toxicity test indicates the fabricated rGO is completely biocompatible, while the composite conductive hydrogel showed slightly toxic effect to the fibroblast cells over the concentration range examined. This may also be caused by the high concentration of I2959 utilized to crosslink the hydrogel. However, the negative effect was observed for two days, afterward, cells tended to proliferate again. Furthermore, the functionality of the released nano-particles was examined under laser scanning confocal microscope, the confocal graphs showed most of the released nano-particle successfully bonded to cell membrane while some of the nano-particles were observed in the cytoplasm of the cells. This indicates the released nano-particles remained its outer structures, as the positively charged outer arginine layer attached to the negatively charged cell membrane. What is

more, western blot experiment was conducted to further confirm the protein silence effect of the *siRNA* drug, the amount of SPARC protein of the control group cells (treated with released scramble nano-particles) is almost 40% more in the amount of the experiment group cells (treated with released *siRNA* nano-particles). This further proved the electric pulse did not affect the particles encapsulated in the conductive hydrogel and in conclusion, the hydrogel could not only be patterned to be used as a conductive hydrogel scaffold but also has the potential to use as safe therapeutic particle carriers, delivery the agents in site for further tissue regeneration purpose.

The important contributions of this work includes the following:

1. Development of new 3D printing system for hydrogel scaffold fabrication with the aid of HL technology. The system were shown to increase the printing speed significantly and largely improved the fabrication efficiency.
2. Investigation of the printer's printing ability with varied experiment parameters.
3. Development of novel biocompatible conductive hydrogel with modified rGO and PEGDA. The hydrogel was shown to be able to transit electrical signal and release incorporated nano-particles under electrical field control.
4. Investigation of *in vitro* release behavior of the conductive composite hydrogel under electrical field control. The particle release rate was found to be dependent upon the intensity of the electrical field applied and the application time.
5. Evaluation of the effect of electrical field on hydrogel network structure. Evaluations of the cell protein knock down effect of the released nano-particles.

5.2 Future Work

Towards providing a better platform for tissue engineering studies, continuous exploration in the tissue scaffold fabrication and particle release from the scaffold is expected. Specifically, the future research work could be focused on several aspects: 1. Study on the hydrogel crosslinking behavior to create more complex and more defined hydrogel scaffold. 2. *In-vivo* study of the conductive hydrogel particle release. 3. Combine the functional conductive hydrogel with the ultra-fast printing system to print electrical-stimuli responsive hydrogel scaffold. 4. Conductive hydrogel scaffold for cardiac tissue engineering. 5. Sustainability of the conductive hydrogel scaffold after electric treatment.

Many studies have proved high porosity is an essential requirement for the tissue scaffold [253], based on the two-layer woodpile structures, additional layers to the current scaffold will be added to form more complex 3D structures. The complexity of the photochemical reaction remained to be a challenging factor in order to print multiple layers structures. The tunable parameters during the printing process such as chemical concentration, illumination time for each layer and the delay time between each separated layers will all affect the integrity and the quality of the structure printed. Thus, the establishment of comprehensive simulation of the PEGDA hydrogel crosslinking behavior is required. The simulated crosslinking behavior of the radical bonding, propagation and termination of the hydrogel can serve as a guideline for the future multiple layer experiment parameter selection.

Secondly, *in vitro* toxicity test of the conductive hydrogel indicates the hydrogel shows slight toxic effect to the fibroblast cells under certain concentration. Whether the hydrogel could be applied to *in vivo* model still remains a question. Thus, *in vivo* test will be conducted in the future to confirm its biocompatibility in animal. At the same time, other non-toxic photo-initiator will also be explored to reduce the toxicity of the polymer.

The potential application for the combination of conductive hydrogel and our new 3D printing system can be specialized in the field of cardiac tissue engineering. Myocardial infarction (MI) and heart diseases have been a big threat to human kinds, and is affecting mortality rates worldwide. Heart transplant remains to an ideal solution to the patient. However, the shortage of donors, and also the strict transplanting safety requirement largely limit the survival rates of the patients. Nowadays, *in-vitro* cardiac tissue engineering has been a challenging research topic due to the strict electro-mechanical property requirement for the tissue matrix. The synchronized movement of the cardiomyocytes requires complex electrical connectivity among each individual cardiomyocytes. The heart muscles, which make up the cardiomyocytes, are composed of highly aligned of multiple layers [59], the orientation of these heart muscles changes from layer to layer to enable the twist behaviors when it contracts. What is more, there are extensive vascular endothelial cells among the cardiomyocytes to transport nutrients and form the blood vessels [85]. Overall, to conduct cardiac tissue engineering, there is a need to fabricate a suitable shaped, electrical stimuli-responsive tissue scaffold with multiple layers. Our ultra-fast printing system could potentially be used to fast print multi-layer conductive hydrogel scaffold to provide required environment for the differentiation of the cardiomyocytes.

List of Publication

1. **Kan Hu**, Jianing An, Yangfei Tan, Andrea Pavesi, Hayden Taylor and Yong Jin Yoon. Ultra-fast, One-step, Three-dimensional Printing of Hydrogel Scaffold. Journal of Biofabrication. *Manuscript in submission*.
2. **Kan Hu**, Yangfei Tan, Jianing An, Alice Ng Jie Ying, Kim Young Jin and Yong Jin Yoon. Photopatternable, “Smart” Conductive Hydrogel for Controllable Drug Release Application. Journal of Macromolecular Bioscience. *Submitted, in revision*.
3. **Kan Hu**, Andrea Pavesi and Yong Jin Yoon. Two-wavelength, Photo-initiation and Photo-inhibition Competing for Selective Modification of Hydrogel Porous Microstructures. Journal of International Precision Engineering and Manufacturing. *Submitted, in revision*.
4. Benjamin Ho, Abhinay Mishra, **Kan Hu**, Jianing An, Kim Young Jin and Yoon Yong Jin. 3D Laser Lithography for Cell Biology Applications. Journal of ACS Biomaterial Science and Engineering. *Submitted, in revision*.
5. Yong Jin Yoon, Lewis Zhang, Benjamin Ho, **Kan Hu**, Jihong Hwang and Seung Ki Moon, Design and Fabrication of Open Cell Structure with Various Porosity Using 3D Additive Manufacturing. Journal of Mechanical Science and Technology. 1738-494, 2013.

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