DEVELOPMENT OF A VISIBLE LIGHT STEREOLITHOGRAPHY-BASED BIOPRINTING SYSTEM FOR TISSUE ENGINEERING

by

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Abstract

Stereolothography-based bioprinting has been considered as a promising solution to generate cell-laden biomaterials for tissue engineering. However, most of the stereolithography-based bioprinting systems employed ultra-violet light to solidify the bioink, a combination of biomaterials and cells. The illumination of ultra-violet light can induce DNA damage and cell cancerization. Therefore, it is safer to utilize non-harmful visible light source for stereololithography-based bioprinting. This thesis presents the design of a simple, low-cost visible light based stereolithography bioprinting system, as well as two novel bioinks supporting visible light solidification. The key features of the developed stereolithography bioprinting system, including the resolution and printing time, were tested. It is found that the low-cost system could reach 60 μm resolution and the printing time for a 100 μm thick layer is less than 4 minutes. The two novel bioinks, named PEGDA-GelMA and GelMA, were characterized to show their mechanical properties and biological compatibility. The PEGDA-GelMA is non cell-adhesive, but with better controllability in its stiffness. The GelMA is relatively soft but cell-adhesive. The system and materials were utilized together in the bioprinting process of NIH-3T3 fibroblast cells. Experimental results show that the cell viability was greater than 85% right after printing. The cells could grow in the bioinks properly for at least five days, proving the feasibility of developed bioprinting solution. Taken together, the developed bioprinting system provides a low-cost visible light stereolithography solution and has the potential to be widely used in tissue engineering applications.
Preface

The studies presented in this thesis are the original work of the author. The research was conducted under the supervision of Dr. Keekyoung Kim at the Integrated Bio-Micro/Nanotechnology Laboratory in the School of Engineering at UBC Okanagan Campus.

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- Chapter 4 has been under review as a research paper.
- Some parts of section 5.2 have been under review as a book chapter.
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<th>Definition</th>
</tr>
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<tr>
<td>Calcein AM</td>
<td>Calcein acetoxymethy</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DMA</td>
<td>Dynamic mechanical analyzer</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified Eagle medium</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>Eosin Y</td>
<td>Eosin Y disodium salt</td>
</tr>
<tr>
<td>EthD-III</td>
<td>Ethidium homodimer III</td>
</tr>
<tr>
<td>GelMA</td>
<td>Gelatin methacrylate</td>
</tr>
<tr>
<td>HA</td>
<td>Hydroxyapatite</td>
</tr>
<tr>
<td>HUVEC</td>
<td>Human umbilical vein endothelial cells</td>
</tr>
<tr>
<td>MEMS</td>
<td>Micro-electromechanical system</td>
</tr>
<tr>
<td>NVP</td>
<td>1-vinyl-2 pyrrolidinone</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PCL</td>
<td>Poly (ε-caprolactone)</td>
</tr>
<tr>
<td>PEG</td>
<td>Poly (ethylene glycol)</td>
</tr>
<tr>
<td>PEGDA</td>
<td>Poly (ethylene glycol) diacrylate</td>
</tr>
<tr>
<td>PEGDMA</td>
<td>Poly(ethylene glycol) dimethacrylates</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscope</td>
</tr>
<tr>
<td>SLA</td>
<td>Stereolithography</td>
</tr>
<tr>
<td>SMC</td>
<td>Smooth muscle cells</td>
</tr>
<tr>
<td>TEA</td>
<td>Triethanolamine</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra-violet</td>
</tr>
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</table>
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Dedication

Dedicated to

My father, Zengjin Wang

My mother, Na Jin

My undergraduate supervisor, Dr. Xia Xiao

And my master supervisor, Dr. Keekyoung Kim
Chapter 1: Introduction

Organ shortage has become more and more problematic in North America. According to the statistics reported by the U.S. Department of Health & Human Service [1], there are approximately 120,000 people waiting for organ transplants. However, less than 20% of them can receive organs for saving their lives. In the last ten years, the number of available donor organs has not significantly increased while the demand for donor organs kept increasing. The deficit of organ donation, as shown in the statistics, is unlikely to be met by a supply of transplantable donor organs that has stagnated over the last decade [2]. At the early stage of research, cell transplants rather than organ transplants were implemented to replace damaged tissues. Since isolated cells can proliferate to reach higher numbers in vitro, only a small number of donor cells were required for initial cell transplants. However, numerous studies have shown that isolated cells could not form functional tissues by themselves [3]. Most of primary cells require specific cellular microenvironments to proliferate and become a tissue [4]. Therefore, tissue engineering, which is an interdisciplinary research area using a combination of cells, biomaterials, and engineering methods to generate artificial functional tissues and organs [5], has emerged to alleviate the organ shortage problem [6].

1.1 Tissue Engineering and Biofabrication

In tissue engineering, cells and biomaterials are combined together into temporary scaffolds to form a proper microenvironment for so-called artificial tissues using various processes, which can be classified as either top-down or bottom-up methods [7]. In the top-down method, cells are seeded homogeneously in the biomaterials to resemble artificial tissues. However, the method is difficult to control extra extracellular matrix (ECM) microenvironments accurately due to
inhomogeneity [7]. Without the proper ECM cells are not able to function as a tissue. On the contrary, bottom-up method aims to build up tissues brick-by-brick and layer-by-layer using microfabrication techniques. The distribution of cells can be well-controlled at the micrometer scale, which has been proven to significantly improve the functionality of tissue scaffolds [8]. Numerous breakthrough approaches in the bottom-up method have been carried out, including the microengineered organ-on-a-chip platforms to mimic the tissue level function in vitro [9], high yield rate differentiation of stem cells [10], and complex heterogeneous tissues [11]. Currently, many researchers are interested in biomaterials and biocompatible microfabrication methods in the bottom-up tissue engineering.

Recently, biocompatible microfabrication methods have become a new interdisciplinary research topic named as biofabrication. Biofabrication was defined in early 2016 as ‘the generation of biologically functional products with structural organization from living cells, bioactive
molecules, biomaterials, cell aggregates such as micro-tissues, or hybrid cell-material constructs, through bioprinting or bioassembly and subsequent tissue maturation processes.’ [12]. As given in the definition, the biofabrication aims to construct functional tissues with biomimetic structures of cells, biomaterials, and other factors using engineering methods. Generally, there are two methods (i.e., bioprinting and bioassembly) used in the biofabrication.

On the one hand, the bioprinting is a kind of biocompatible additive manufacturing methods. Guillemot et al. defined the bioprinting as ‘the use of computer-aided transfer processes for patterning and assembling living and non-living materials with a prescribed 2D or 3D organization in order to produce bioengineered structures serving in regenerative medicine, pharmacokinetics and basic cell biology studies.’ [13]. The key point in this definition is the computer-aided transfer processes. Therefore, similar to other transfer processes, such as 3D printing or photolithography, desired complex structures are achieved by directly one or more bioprinting processes. On the other hand, the bioassembly aims to fabricate desired structures with pre-formed building blocks. Groll et al. defined the bioassembly as ‘the fabrication of hierarchical constructs with a prescribed 2D or 3D organization through assembly of pre-formed cell-containing fabrication units generated via cell-driven self-organization or through preparation of hybrid cell-materials building blocks, typically by applying enabling technologies, including microfabricated molds or microfluidics.’ [12]. Therefore, the bioassembly is a kind of indirect, multi-steps fabrication processes. The bioassembly offers a good control of the cellular microenvironments [14] and has successfully generated many functional tissue structures, such as microfibers [15], [16] and microbead-based macro tissues [17]. However, the complicated multi-steps fabrication procedures and the complex handling process limit the wide applications of the
bioassembly. Hence, bioprinting has attracted more and more research interests in recent years due to its advantage of one-step fabrication, full automation, and simple process [18].

1.2 Bioprinting Techniques

In bioprinting, small units of the mixture of cells and biomaterials are deposited at micrometer resolution to fabricate artificial tissues. Unlike the conventional 3D printing technique that has been used to print cell-free scaffolds using in surgery [19], bioprinting is capable of depositing living cells in the desired shapes to fabricate living tissues. A typical workflow of artificial tissues using bioprinting is given in Figure 1.1. First of all, cells are isolated from the human body and cultured in vitro to have a large number of cells for tissue fabrication. Subsequently, the cells are mixed with biomaterials and printed to form a biomimetic cell-laden scaffold. The scaffold is incubated several weeks to generate artificial tissues before the transplantation.

Figure 1.2 Schematic of inkjet bioprinting (Adopted from [11])
Comparing to other biocompatible microfabrication techniques, such as microfluidics and micromolding, the bioprinting offers more versatile, rapid, scalable, and cost-effective way to fabricate cell-laden biomaterial scaffolds. With these advantages, the development and applications of bioprinting have greatly increased in the last ten years. However, there is no one-for-all solution for the printing of complex, heterogeneous tissues to date. Most of the bioprinting systems are based on one of the following techniques: inkjet, extrusion, laser-assisted or stereolithography (SLA).

1.2.1 Inkjet Bioprinting

Inkjet bioprinting is the first bioprinting technology introduced by Tao et al. in 2005 [20] and very similar to the conventional 2D inkjet printing. A biomaterial mixed with cells, which is called as a bioink, is stored in the ink cartridge. The cartridge is then connected to a printer head and acts as a printing source for the computer controlled printing process. During printing, the printer head is deformed by a piezoelectric actuator and squeezed to generate droplets at a controllable size, as shown in Figure 1.2. The advantages of inkjet printing include: (1) relatively high cell viability (usually from 80% to 90%, as reported by many research studies [20]–[23]), (2) relatively low cost as a result of similarity to a conventional printer. However, because current inkjet printer heads are based on microelectromechanical system (MEMS), the printer head is only capable of generating discontinuous droplets, which results in relatively poor 3D structures in vertical direction [18]. In addition, since the deformation of MEMS-based printer head is relatively small, it is difficult for inkjet printing to squeeze out high viscosity materials [11]. High cell density increases the average viscosity of bioinks, resulting in clogging of the printer head [24]. The
limitation of printing true 3D structures and clogging problem significantly hinder the applications of the inkjet bioprinting.

1.2.2 Extrusion Bioprinting

Extrusion bioprinting has been emerged as the solution to overcome the limitation of printing 3D structures in the inkjet bioprinting. It uses either an air-force pump or a mechanical screw plunge to apply continuous force to extrude bioinks, as shown in Figure 1.3. The continuous force results in the generation of cylindrical lines rather than droplets. The cylindrical lines offer a much improved 3D structure of bioinks [18]. However, due to the large mechanical forces applied during extrusion, the cell viability is decreased dramatically (usually in-between 40% to 80%). Another problem of extrusion printing is the high cost of the pump-based dispenser.

Figure 1.3 Schematic of extrusion bioprinting (Adopted from [11])
Laser-assisted bioprinting originated from laser-induced transfer technology [25]. Figure 1.4 shows a schematic of laser-assisted bioprinting. The critical part of the laser-assisted printing system is a donor layer that responds to laser stimulation. The donor layer comprises a ‘ribbon’ structure containing an energy-absorbing layer (usually titanium or gold [26]) on the top and a layer of bioink solution suspended on the bottom. During printing, a focused laser pulse is applied to stimulate a small area of the absorbing layer. This laser pulse vaporizes a portion of the donor layer, creating a high-pressure bubble at the interface of the bioink layer and propelling the suspended bioink. The falling bioink droplet is collected on the receiving substrate and subsequently crosslinked. Compared to inkjet printing, laser-assisted printing can avoid direct contact between the dispenser and the bioink, which does not cause mechanical stress to the cells. As a result, the cell viability is very high (usually higher than 95%). However, to have an accurate control of the laser pulse, most of the laser-assisted bioprinting system employed the expensive
femtosecond laser. Also, many parameters affecting droplet size and printing quality are not systematically characterized. Therefore, laser-assisted bioprinting is less controllable, compared to other printing techniques.

1.2.4 Stereolithography-based Bioprinting

As one of the most popular techniques in additive manufacturing, stereolithography (SLA) has also been modified for bioprinting purposes. The SLA-based bioprinting uses ultra-violet (UV) light to selectively solidify a photo-sensitive bioink in a layer-by-layer fashion, as shown in Figure 1.5. SLA uses an array of digital micromirrors to control the light intensity applied to each small area. The digital micromirror array used in beam projectors is commercially available from Texas Instrument. SLA-based bioprinting has several advantages over nozzle or print head-based

![Figure 1.5 Schematic of stereolithography bioprinting (Adopted from [11])](image-url)
bioprinting. First of all, no matter how complex pattern in one layer is, the printing time remains the same because the entire pattern of one layer is projected over the printing plane at a time. In addition, the SLA-based system requires only Z-axis stage, which significantly simplifies the control of bioprinting process. Moreover, the cell viability after SLA-based bioprinting is high (more than 90%) because no actual force is applied to cells during printing. The drawback of SLA-based bioprinting is the potentially harmful effect of UV light on cells, which will be discussed in Section 1.4 in detail. Table 1.1 summarized the key features of three different types of bioprinting techniques. Considering cost, resolution, integrity of 3D structure, and cell viability, SLA-based bioprinting is a more promising solution than inkjet- and extrusion-based bioprinting for building complex human tissue structures.

Table 1.1 Comparison of four types of bioprinting techniques.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Inkjet</th>
<th>Extrusion</th>
<th>Laser-assisted</th>
<th>Stereolithography</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cost</td>
<td>Low</td>
<td>High</td>
<td>Extremely High</td>
<td>Low</td>
<td></td>
</tr>
<tr>
<td>Cell viability</td>
<td>&gt; 85%</td>
<td>40 – 80%</td>
<td>&gt; 95%</td>
<td>&gt; 90%</td>
<td>[11], [18]</td>
</tr>
<tr>
<td>Quality of vertical structure</td>
<td>Poor</td>
<td>Good</td>
<td>Fair</td>
<td>Good</td>
<td></td>
</tr>
<tr>
<td>Print speed</td>
<td>Fast</td>
<td>Slow</td>
<td>Medium</td>
<td>Fast</td>
<td></td>
</tr>
</tbody>
</table>
1.2.5 Bioinks

In addition to the printing techniques, bioinks are also one of the major factors in the bioprinting process. Bioinks typically consist of a biomaterial and biological cells, which forms the cellular microenvironment of printed cells. In general, the requirements of a bioink are to be printable, crosslinkable, and biocompatible [11]. Printability of the bioink is largely associated with its viscosity. If a material is too viscous, it may not be printable using the inkjet bioprinting. Crosslinkability is the condition that the printed liquid form of materials, which is a so-called pre-polymer solution, should be able to form a solid network. For general biomedical applications, biocompatibility is required since the material should not be harmful to cells and have the ability to provide proper cell binding sites [27].

1.2.5.1 Hydrogels

Hydrogels are the most widely used biomaterials for the bioinks because of its advantage of closely mimicking in vivo microenvironments of cells [28]. From the perspective of hydrogel design, there are two types of hydrogels: natural polymer-based hydrogels and synthetic polymer-based hydrogels [29]. Natural hydrogels include polymers existing in ECM components, such as gelatin, collagen, laminin and fibronectin, as well as other natural hydrogels such as alginate, chitosan and silk fibroin. Interactions between natural hydrogels and cells have been well investigated [29]. Unlike natural hydrogels, synthetic hydrogels are made through chemical synthesis process and typically more controllable in terms of their chemical and mechanical properties [30]. Their interactions with and effects on cells, however, have not yet been studied systematically [29]. Both natural hydrogels and synthetic hydrogels are widely used in bioprinting researches [11].
Hydrogel pre-polymers are in a liquid form before solidification. It can form a network of polymer chains to become solidified gels with the excitation by temperature, chemicals, or illumination [31]. The solidification process of the hydrogel is called as the crosslinking which happens during bioprinting process. In general, the inkjet and extrusion bioprinting are compatible with thermal, chemical, and photo-crosslinking methods. The SLA-based bioprinting can only be done by the photo-crosslinking due to its light-based printing nature. Among all crosslinking methods, the photo-crosslinking has become the most widely used method in recent years [29]. The major advantages of the photo-crosslinking include the fast crosslinking rates (usually from several seconds to a few minutes) at a physiological temperature, minimal heat production, and minimal invasion to the fabricated microstructures in situ [32]. The minimal invasion to the in situ structures is especially critical for bioprinting because it allows the formation of complex and accurate shapes of bioinks that can adhere and conform to functional tissue structures.

After crosslinking, hydrogels turned into a solid network to provide a suitable 3D cellular microenvironment for cell growth and proliferation. To offer cells with a stable environment for proliferation, hydrogels should maintain sufficient mechanical properties [33]. These mechanical properties include strain, shear stress, compressive modulus, and mass swelling ratio. Mechanical properties are considered to be highly essential for soft tissues, such as cartilage and skin, as the function of such tissues mainly relies on their mechanical properties [34]. In addition, it is desirable for implanted tissues to eventually fuse with surrounding in vivo tissues. As a result, hydrogel scaffolds are degraded or integrated with the in vivo ECM environment. Therefore, hydrogels with a controllable degradation rate are highly desired [18].
1.2.5.2  Cells

Mammalian cells with a potential to proliferate in vitro are suitable for bioprinting and one of the important parts of bioinks. To build a highly biomimetic tissue or organ on the macro scale, the bioprinted cells for bioprinting are required to consider how closely the bioprinted cells can mimic the physiological state of cells in vivo, and to what degree the bioprinted cells can maintain or develop their in vivo function under optimized microenvironments [18]. Artificial tissues can be formed by printing either primary cells with supportive cells directly or printing stem cells for further differentiation [11]. Direct printing of primary cells can rapidly increase the complexity of bioprinting since multiple cells in different bioinks can be printed in a well-controlled manner. Printing stem cells usually are able to reduce the total amount of bioinks used for printing. However, it adds complications in the post-printing process because the stem cells need to be differentiated into primary cells after printing.

In practical bioprinting applications, researchers choose cells for printing depending on the target tissues that are needed to be generated. For example, to generate vessels, human umbilical vein endothelial cells, which are the cells to form the inner wall of blood vessels can be mixed with a biomaterial to make a bioink for printing blood vessels [35]. Because not all types of cells can regenerate (i.e., cardiac muscle cells), stems cells with the ability to differentiate into the desired cells are the most promising cell sources for the regeneration of various types of tissues in bioprinting applications [36].

1.3  Applications of Bioprinting

As discussed in Section 1.1, bioprinting is considered as a biocompatible microfabrication technique for tissue engineering applications and has advantages in fabricating complex tissue
structures. Therefore, the main application of bioprinting is to generate artificial tissues, which have the potential for clinical transplantations illustrated in Figure 1.1. Over the last few years, bioprinting has successfully generated groups of tissues, including vessel [37], bone [38], cartilage [39], skin [40], tumor [41], neuron [42], muscle [43], and adipose [44]. These bioprinted tissues retain cell viability and function. Table 1.2 provides several bioprinting studies of various tissues. Recently, researchers have fabricated more complex and heterogeneous tissues with various cells and biomaterials, which closely mimics in vivo tissue structures [35], [45], [46]. Kolesky et al. successfully fabricated a centimeter thick vascularized tissue with 12 micro vessels inside [46]. Kang et al. studied in vivo maturation and the fusion process after the implantation [47]. They observed the vascularization and neuron regeneration after two weeks of implantation. Two more weeks later, they obtained a functional bioprinted skeletal muscle, which responded to the electrical stimulation of the nerves and was able to generate mechanical forces. This study further proved the feasibility of using bioprinting to generate functional tissues for clinical applications. In addition to artificial tissues, bioprinting was capable of building in vitro microscale tissues for drug screening [48] and quantitative biology [49]. In view of high controllability and resolution, bioprinting is also promising to build high-throughput platforms on a microfluidic chip for screening drugs, characterizing cells, and optimizing stem cell differentiation [50].
Table 1.2 Representative bioprinting studies.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Cell sources</th>
<th>Materials</th>
<th>Printing method</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vessel</td>
<td>Smooth muscle cells (SMC)</td>
<td>Carbon nanotube encapsulated alginate</td>
<td>Extrusion</td>
<td>[51]</td>
</tr>
<tr>
<td></td>
<td>SMC and aortic valve leaflet interstitial cells</td>
<td>Gelatin and alginate</td>
<td>Extrusion</td>
<td>[52]</td>
</tr>
<tr>
<td></td>
<td>Human umbilical vein endothelial cells (HUVEC)</td>
<td>PEGDA, Matrigel, fibrin gel, alginate, agarose, and GelMA</td>
<td>Extrusion</td>
<td>[35], [53]</td>
</tr>
<tr>
<td></td>
<td>Rat heart endothelial cells</td>
<td>Alginate</td>
<td>Extrusion</td>
<td>[54]</td>
</tr>
<tr>
<td></td>
<td>Ea.hy926 endothelial cells</td>
<td>Nano-hydroxyapatite (n-HA)</td>
<td>Laser-assisted</td>
<td>[55]</td>
</tr>
<tr>
<td></td>
<td>L929 Fibroblasts, mouse endothelial cells and human mesenchymal stem cells</td>
<td>Acrylated hyaluronic acid-PEG, and Matrigel</td>
<td>Inkjet</td>
<td>[56]</td>
</tr>
<tr>
<td></td>
<td>HUVEC</td>
<td>GelMA</td>
<td>SLA</td>
<td>[57]</td>
</tr>
<tr>
<td>Bone</td>
<td>Mouse osteoblastic cells</td>
<td>n-HA</td>
<td>Inkjet</td>
<td>[58]</td>
</tr>
<tr>
<td></td>
<td>MG-63 cells</td>
<td>Alginate</td>
<td>Extrusion</td>
<td>[59]</td>
</tr>
<tr>
<td></td>
<td>Human osteoprogenitor cells</td>
<td>n-HA</td>
<td>Laser-assisted</td>
<td>[60]</td>
</tr>
<tr>
<td>Cartilage</td>
<td>Patient’s cartilage</td>
<td>poly(ethylene glycol) dimethacrylates (PEGDMA)</td>
<td>Inkjet</td>
<td>[61]</td>
</tr>
<tr>
<td></td>
<td>Minced cartilage cells</td>
<td>Poly (ε-caprolactone) (PCL), and fibrin-collagen hydrogels</td>
<td>Inkjet</td>
<td>[39]</td>
</tr>
<tr>
<td></td>
<td>Equine chondrocytes and mesenchymal stromal cells (MSCs)</td>
<td>PCL, GelMA, and GelMA-gellan hydrogels</td>
<td>Extrusion</td>
<td>[62]</td>
</tr>
<tr>
<td></td>
<td>Human meniscus cells</td>
<td>GelMA</td>
<td>SLA</td>
<td>[63]</td>
</tr>
<tr>
<td>Skin</td>
<td>3T3Fibroblast, HaCaT keratinocytes</td>
<td>Collagen</td>
<td>Laser-assisted</td>
<td>[40]</td>
</tr>
<tr>
<td>Neuronal tissue</td>
<td>Mouse bone marrow stem cells</td>
<td>Collagen, and agarose</td>
<td>Extrusion</td>
<td>[42]</td>
</tr>
<tr>
<td></td>
<td>Embryonic stem cells</td>
<td>N/A</td>
<td>Inkjet</td>
<td>[64]</td>
</tr>
<tr>
<td>Skeletal muscle</td>
<td>C2C12 mouse myoblasts</td>
<td>Polyurethane, and PCL</td>
<td>Extrusion</td>
<td>[43]</td>
</tr>
<tr>
<td></td>
<td>C2C12 mouse myoblasts</td>
<td>Alginate, and gelatin</td>
<td>Extrusion</td>
<td>[65]</td>
</tr>
<tr>
<td>Tumor</td>
<td>Hela cells</td>
<td>Gelatin-alginate-fibrinogen hydrogel</td>
<td>Extrusion</td>
<td>[41]</td>
</tr>
<tr>
<td>Adipose tissue</td>
<td>Adipose derived stem cells</td>
<td>Alginate</td>
<td>Laser-assisted</td>
<td>[44]</td>
</tr>
</tbody>
</table>
1.4 Existing Stereolithography-based Bioprinting Systems

Gauvin et al. reported a custom built UV light-based (365 nm in wavelength) SLA bioprinting system [57]. As reported in a group of studies [57], [63], [66], [67], the bioprinting system was capable of printing 3D complex cell-laden scaffolds with the resolution down to 50 μm. The upgraded system was able to print nanoparticle-embedded biomaterials with a resolution of 10 μm [68]. Although the developed system is technically sound and has been applied to several studies, the UV light-based SLA bioprinting has a problem as the UV light cause cell damage. It is reported that UV B-band light (wavelength 290 – 320 nm) can induce apoptosis via activating the death receptor CD95 in the cell nuclear DNA [69]. For UV A-band light (wavelength 320 – 400 nm) as shown in Figure 1.6, the UV photons at UV A-band can induce the DNA damage which may contribute to the cancerization of cells [70], [71]. Therefore, the UV light-based SLA bioprinting is less biologically compatible due to the high risk of cancerization and DNA damage of cells.

Figure 1.6 Schematic of DNA damage by UV illumination (Copyright David Herring NASA's Earth Observatory)
To avoid the harmful effects of UV light, Lin et al. modified a commercially available SLA printing system for bioprinting applications [72]. The system used a 405 nm blue light for printing adipose-derived stem cell-laden scaffolds with the minimal feature of 100 μm. However, the 405 nm blue light is very close to UV A-band (320 – 400 nm). It has been reported that the blue light could decrease cell viability significantly [73], [74]. Therefore, the blue light cannot be the alternative solution of UV light for the SLA-based bioprinting. For 3D bioprinting applications, the SLA-based 3D printing system requires much safer light sources which are in visible light (longer wavelength) ranges and non-harmful to cells. However, modifying commercialized SLA-based 3D system with a safe light source may be an expensive solution.

Although non-UV based photo-crosslinking methods are highly desired for the SLA-based 3D bioprinting system, little research has been done in the development of visible light crosslinkable hydrogels. Bahney et al. comprehensively studied visible light crosslinkable poly (ethylene glycol) diacrylate (PEGDA) hydrogels and proved its biocompatibility for 14 days [75]. However, this study has not been applied to 3D bioprinting, and the compatibility of visible light crosslikable hydrogels with the SLA-based 3D bioprinting system is unknown.

1.5 Research Objectives

The SLA-based 3D bioprinting system is one of competitive 3D bioprinting techniques in terms of printing speed and cell viability. However, the direct applications of the SLA-based printing systems in tissue engineering were limited due to the non-biocompatibility of UV light used in the printing system. To date, no visible light has been used for the SLA-based bioprinting system. Also, it is highly desired to develop a new bioink based on visible light crosslinkable hydrogels. To test the feasibility of SLA-based bioprinting using the visible light, a new bioprinting system
with visible light crosslinkable hydrogels need to be developed. Therefore, the objectives of this thesis are:

1. Design a SLA-based 3D bioprinting system using the visible light.

2. Test the feasibility of the developed system for printing visible light crosslinkable hydrogels.

3. Develop novel visible light crosslinkable hydrogels with high biocompatibility for the SLA-based 3D bioprinting.

To achieve the objectives, a simple, high-resolution visible light SLA-based bioprinting system was designed and tested. The system was utilized to print several customized patterns to characterize its printing capability and minimum feature size. Two novel visible light crosslinkable bioinks, which were poly (ethylene glycol) diacrylate – gelatin methacrylate (PEGDA-GelMA) hybrid bioinks and high concentration gelatin methacrylate (GelMA) bioinks with different properties, were developed. The compatibilities of the bioinks with the developed system were verified by printing several patterns. The mechanical properties and microstructures of both bioinks were examined by using several material characterization techniques. The biocompatibilities of the bioinks were verified by cell encapsulation experiments.

1.6 Chapter Outline

This thesis describes the development and characterization of the novel SLA-based bioprinting system, as well as the visible light crosslinkable biomaterials. Chapter 1 discusses backgrounds on tissue engineering and 3D bioprinting. Several bioprinting techniques are reviewed, and the existing SLA-based 3D bioprinting systems are discussed in detail. Chapter 2 describes the design and integration of novel SLA-based 3D bioprinting system. Details regarding the system setup
including a beam projector and temperature control mechanism are described. Also, the bioprinting capability and printed samples are evaluated. Chapter 3 presents the development of PEGDA-GelMA hybrid bioinks. The design strategy of various hybrid materials, the details of material characterization results, and experimental results of 3D bioprinting are discussed. Chapter 4 describes the development of GelMA bioinks for the SLA-based 3D bioprinting. The details of material characterization and bioprinting experiments are included. Chapter 5 gives a short conclusion and a future perspective of the SLA-based bioprinting research, along with a list of my referred contributions.
Chapter 2: Development of a Visible Light Stereolithography-based 3D Bioprinting System

Existing SLA-based bioprinting systems used UV lights as the light source for crosslinking light sensitive polymer resin. To adopt visible light crosslinkable polymer materials for 3D bioprinting, it is required to develop a novel SLA-based 3D bioprinting system using a visible light. Also, the system should be able to print samples with acceptable resolution and offer a biocompatible printing process to maintain high cell viability after printing. This chapter presents details regarding the design and system integration of a low-cost visible light SLA-based 3D bioprinting system. The minimum feature size and the feasibility of bioprinting using the developed system are determined by printing various samples of a visible light crosslinkable hydrogel.

2.1 System Design

The visible light SLA-based bioprinting system includes four main components, including a beam projector as the light source, a filter system to remove the by-product of infrared light, an acrylic chamber as a supporting frame, and a closed-loop controller to maintain the temperature inside the

![Figure 2.1 Schematic of developed visible light SLA-based bioprinting system.](image)
chamber. Figure 2.1 shows the schematic of the developed system. Based on the input from a computer, the beam projector (Component 1) controls the brightness of each projected pixel and generates an array of white and black lights (Component 2). This array of lights is reflected and focused by the mirror lens-based focusing system (Component 3) and pass through the water filter (Component 4). Through the filtering of the water filter, the harmful infrared light emitted by the beam projector is eliminated. Filtered lights are directly projected onto a photocrosslinkable hydrogel pre-polymer solution (Component 5). Depending on the brightness of the light, the specific areas of the hydrogel forms 3D printed patterns. The SLA-based bioprinting system is a layer-by-layer fabrication method. To fabricate a complex structure in the vertical direction, a new layer of hydrogel pre-polymer solution is added through a syringe pump before the crosslinking process (Component 6). The entire system is supported by the acrylic frame (Component 7).

Figure 2.2 shows the design of a temperature control system. The temperature control inside the printing chamber is achieved by recycling a heat flow from the cooling system of the beam

![Diagram](image)

*Figure 2.2 Working mechanism of the closed-loop temperature control system.*
projector. During projection, the lamp of the beam projector generates excessive heat which is released by a cooling fan continuously. Heat flow from the cooling fan is bypassed through a 6 cm x 6 cm opening with a fan into the acrylic frame chamber where the material is printed. A closed-loop feedback controller is used to achieve temperature control. The temperature sensor on the controller is put near the sample undergoing bioprinting. If the temperature is lower than 36.5 °C (Note that the temperature for cell incubation is 37 °C), the fan is turned on by the controller and the heat flow generated by the projector goes into the chamber. If the temperature is higher than 37.5°C, the fan is turned off. By applying this simple system, the temperature inside the printing chamber can be controlled between 36.5 °C and at 37.5 °C, which maintains a suitable environment for cells to survive during bioprinting.

The selectively-crosslinking mechanism of one layer is illustrated Figure 2.3. Inside the beam projector, there is a microfabricated micromirrors array, which is called a digital micromirror device (DMD). One micromirror represents a pixel of the computer screen. The DMD can control the angle of the mirror inside, which determines the brightness of a light pass through the mirror. Therefore, DMD array is able to control the light intensity of each pixel that is projected. In the DMD array as shown in Figure 2.3, the bright gray color means that the pixels are in white color (refer to the high intensity of light) while the dark gray means the pixels are in black color (refer to the low intensity of light). The light beams generated by the DMD array are passed through the lens system to focus the light beam onto a small area for high-resolution printing. With the help of
this focusing lens system, the beam projector can clearly focus on objectives onto the bottom of the printing chamber. The field of view is approximately 9.6 cm x 5.4 cm, and the pixel size is 50 x 50 µm with the full high definition resolution (1920 x 1080 pixels) of the projector.

The focused light beam is filtered by a water filter which is made by a transparent acrylic container filled with deionized water up to 4 cm height. The reason for introducing the water filter is to protect cells from the heat generated by the infrared light which causes cell death [76]. The infrared light is the by-product of a halogen lamp used in the beam projector. Since water absorbs an emitted heat generated by the light, the water filter was used to investigate the cell response to strong light sources [77], [78]. Here, the water filter is adopted to eliminate the harmful effect of the infrared light on cells. The filtered light beam is projected on the layer of a hydrogel pre-polymer solution. The pre-polymer solution is sensitive to the visible light and can be crosslinked
to form a hydrogel scaffold when exposed to the visible light with a certain intensity. The high-intensity light from the white pixel of DMD array is able to crosslink a visible light crosslinkable hydrogel at a certain period of time while the low-intensity light is not able to crosslink the hydrogel. As a result, the system is able to selectively crosslink patterns in the desired areas of hydrogels for bioprinting.

Figure 2.4 presents the processes to fabricate 3D scaffolds which are based on single layer patterning. After the patterning of one layer, a new pre-polymer solution with controlled volume to have a 100 µm thick layer is added by a syringe pump. Then, another single layer patterning process starts to build up 3D structure until the entire patterning process is finished. The crosslinking time is controlled precisely to ensure that only the newly-added layer is crosslinked while the uncrosslinked area in the previous layer is not significantly affected by a consecutive printing process. Since the projector has a best-focused plane of light, it is worth noting that the

![Diagram](image)

**Step 1**
Add new layer of uncrosslinked solution to cover the pattern.

Move to step 2 to pattern this layer.

**Step 2**
Patterned newly-added layer. The patterning time is controlled precisely to avoid unwanted crosslinking in the bottom layer if any.

If the pattern is unfinished, go step 1, otherwise go step 3.

**Step 3**
The pattern was finished, uncrosslinked hydrogels were removed to release desired pattern.

Figure 2.4 Crosslinking mechanism of multi-layers.
resolution of the pattern becomes worse if the pattern is too thick. In this case, some layer is significantly far from the best-focusing plane, which results in the blurry projected pattern. However, such effect is minor when the thickness of the pattern is less than 5 mm.

2.2 System Setup

Figure 2.5 shows the system setup of 3D bioprinting. A beam projector (HD6510BD, Acer, Taipei, Taiwan), which was connected to the computer, was used as the device for the SLA-based bioprinting. The projector was placed 4 cm away from the reflection mirror. The distance between the mirror and printing sample was around 6 cm. There was a 4 cm thick water filter between the

![Figure 2.5 Photograph of developed visible light SLA-based bioprinting system.](image-url)
mirror and sample to filter the harmful infrared radiation generated as the by-product of the halogen lamp in the projector. A syringe pump (Genie Touch, Kent Scientific, Torrington, CT, USA) was employed to add the bioinks before the crosslinking of each layer. The closed-loop control system was used to control the temperature in the chamber as described in Section 2.1. The total cost of the system is less than CDN$ 2,000 including a beam projector and syringe pump.

### 2.3 Bioprinting Workflow

The workflow of bioprinting system can be divided into three main steps: pre-printing, in-printing, and post-printing, as shown in Figure 2.6. In the pre-printing step, the printing structure is customized by a 3D design software (AutoCAD 2014, Autodesk, San Rafael, CA, USA). The designed structure was saved as an STL (stereolithography) file. The STL file was sliced with a
gap of 100 μm in the Z direction via Freesteel Z-level slicer software (version 1.5, Freesteel, Liverpool, UK). The slices of layers were converted to binary color patterns. The areas need to be crosslinked were in white color. During the printing, sliced binary color images were projected by the beam projector layer-by-layer. The projection time depends on the type of materials and is determined by when specific materials for experiments are used. The mechanism of multi layers patterning has been described in Section 2.1. After the printing, the uncrosslinked pre-polymer solution was removed by pipetting. The printed samples were washed with phosphate-buffered saline (PBS, Life Technologies, Carlsbad, CA, USA) three times to remove the residues of the uncrosslinked solution. Depending on the applications, the samples were further cultured to check cell proliferation or stained by the food dye to visualize the pattern.

2.4 Characterization of System Performance

To examine the performance of the developed system, a visible light crosslinkable hydrogel was used to print samples. Bahney et al. reported a biocompatible visible light crosslinkable hydrogel based on Eosin Y to initiate photocrosslinking process [75]. Therefore, this thesis adopted the Eosin Y-based visible light crosslinkable hydrogel for preparing a bioink and characterizing bioprinted samples. Details regarding the crosslinking mechanism of the Eosin Y-based photoinitiator are discussed in Section 3.1. The pre-polymer solution was the mixture of 10% w/v poly (ethylene glycol) diacrylate (PEGDA, molecular weight: 700 Da), 0.01 mM Eosin Y disodium salt (Eosin Y), 0.1% w/v triethanolamine (TEA), and 37 nM 1-vinyl-2 pyrrolidinone (NVP) in PBS solvent. All the materials were purchased from Sigma-Aldrich (St. Louis, MO, USA).
The developed bioprinting system was used to print UBC logo and 4-by-4 mesh patterns to investigate the feasibility of the system. Both patterns had eight layers and required 16 minutes in total for printing (2 minutes per each layer). After printing, the patterns were colored with a red food dye to visualize the pattern. As shown in Figure 2.7, the patterns were clear and intact, proving the feasibility of the developed system. To determine the resolution of the system, a mesh pattern with different line widths (1 pixel, 2 pixels, and 5 pixels) was printed. The width of 1 pixel line determines the minimum resolution of the system. The patterns were examined under an inverted optical microscope (ToupTek Photonics, Hangzhou, China).

Figure 2.7 Hydrogel patterns fabricated with the developed bioprinting system. Printed (a) UBC logo and (b) 4x4 mesh pattern on a petri dish. Scale bar = 1 cm. A zoon-in view of (c) UBC logo and (d) 4x4 mesh pattern. Scale bar = 2 mm. (Adopted from [85])
As shown in Figure 2.8, the resolution of the system was around 50-60 μm. This result matched with the theoretical calculation based on the field of view, which described in Section 2.1. In addition, the width of 2 pixel lines was approximately 100 μm, and the width of 5 pixel lines was around 500 μm. Therefore, the relationship between the width of the pattern and the number
of pixels were linear, which provides a guideline for the design of patterns. The notable texture pattern on the surface of PEGDA resulted from the crosslinking process was shown in Figure 2.8. Lin et al. also observed a similar surface texture on 4000 Da PEGDA after patterning [72]. The printing results demonstrate that the developed bioprinting system could print hydrogels with micrometer resolution. In addition, the biocompatibility of the printing process is one of the important factors for bioprinting since cells need to be alive and maintain their functions for tissue engineering applications. To verify the biocompatibility of the system, the PEGDA pre-polymer solution with the Eosin Y-based photoinitiator were mixed with NIH 3T3 fibroblasts (cell density: 2 x 10^6 cells/mL) and patterned. Then, the encapsulated cells in PEGDA patterns were cultured in the incubator with Dulbecco's modified Eagle medium (DMEM, Lonza, Basel, Switzerland) with 10% v/v fetal bovine serum (FBS, Life Technologies), and 1% v/v penicillin-streptomycin (Life Technologies). Cell viability was examined right after the printing process using the live/dead cell viability assay (Biotium, Hayward, CA, USA). To stain cells with the live/dead assay, 5 μL of 4 mM calcein acetoxyethyl (calcein AM) and 20 μL of 2 mM ethidium homodimer III (EthD-III) were mixed with 10 mL of PBS to have a 2 μM calcein AM/ 4 μM EthD-III working solution. The samples were washed with PBS twice and incubated with 400 μL of working solution for 60 minutes. Finally, the samples were washed with PBS once and ready for taking the microscope images. Stained samples were then observed under a confocal fluorescence microscope (FV1000, Olympus, Tokyo, Japan). The representative images captured under 10 X objective were given in Figure 2.9.
Two fluorescent channels and one phase contrast channel were combined to count live and dead cells. As shown in Figure 2.9, most of the cells were alive and stained with green color. A few dead cells inside the pattern were found. Therefore, the result demonstrated that cell viability right after printing was high and the bioprinting system was highly biocompatible. It is noted that the hydrogel was also stained with green color, which may be the reason why the molecular weight
of the PEGDA is relatively small (700 Da) and the permeability is not enough to wash the green dye away.

The bioprinting was also conducted without the help of the water filter. The fluorescence image of live/dead stained bioprinting samples is shown in Figure 2.10. There were no live cells in the field of view, which proved the necessity of the water filter in the developed bioprinting system. The previous bioprinting result of PEGDA hydrogel revealed that the SLA-based bioprinting process was biocompatible. However, it was found that the cell viability was dropped down dramatically after 5 days as shown in Figure 2.11. The cell death may be caused by the PEGDA hydrogel.

Figure 2.10 Live/dead assayed bioprinting pattern of PEGDA hydrogel at day 0 without the help of water filter. Red dots indicate dead cells. Scale bar = 100 μm. (Adopted from [85])
Although PEGDA has been used with long-term culturing of stem cells [75], the molecular weight of the PEGDA used in the research was 6000 Da, which is eight times greater than the PEGDA used in this study. According to the study from Zhang et al. [79], the lower molecular weight of PEGDA had better stiffness because of the dense structure. The dense structure, however, can result in the small pore size of the microstructure, affecting the proliferation of cells [80]. Therefore, the pore size of crosslinked PEGDA with 700 Da was examined using a scanning electron microscope (SEM, Mira 3 XMU, TESCAN, Brno, Czech Republic). For taking the SEM images, the crosslinked samples of PEGDA were freeze-dried for five days. Then, the freeze-dried samples were coated with 10 nm thick gold-palladium (Au-Pd) alloy using sputtering.

Figure 2.11 Live/dead assayed bioprinting pattern at day 5. Bright green dots indicate live cells while red dots indicate dead cells. Scale bar = 200 μm.
As shown in Figure 2.12, the microstructure of 10 wt% PEGDA was very dense without pores larger than 5 μm. The pore size of the 10% w/v PEGDA is significantly smaller than the size of cells (~ 20 μm). Considering the facts that most of the biomaterials for cell encapsulation have the pore size greater than 50 μm [80], the small pore size of the PEGDA may contribute to the cell death.

2.5 Chapter Summary

In this chapter, it is demonstrated that the developed SLA-based bioprinting system was able to pattern PEGDA hydrogels using the visible light emitted by a conventional beam projector. The printing speed of a 100 μm thick layer was 2 minutes. The minimum resolution of the system was around 50 - 60 μm as examined by the microscope image. The bioprinting process was proven to be biocompatible through the printing of NIH-3T3 cells encapsulated in the hydrogels and investigation of cell viability right after printing. The developed system provided the safe and
inexpensive 3D bioprinting solutions with the cost lower than CDN$ 2,000, a high-speed, high-resolution to fabricate complex cell-laden scaffolds in 3D for tissue engineering applications. However, the small pore size of the crosslinked PEGDA hydrogel used in this study affected the long-term cell viability significantly. A new hydrogel with larger pore size as a bioink is required for bioprinting and tissue engineering applications.
Chapter 3: Visible Light Crosslinkable Hybrid Bioinks

In chapter 2, a visible light SLA-based bioprinting system has been successfully developed and tested. The visible light crosslinkable hydrogel was adopted from the study reported by Bahney et al. [47] has been proven to be compatible with the developed bioprinting system. However, this hydrogel is not a suitable solution as a bioink for bioprinting and tissue engineering applications because of its poor long-term cell viability [51]. Therefore, this chapter aims to the design and characterization of visible light crosslinkable hybrid bioinks with high long-term cell viability.

3.1 PEGDA-GelMA Hybrid Bioinks

Hutson et al. demonstrated that cell viability was much improved by mixing the PEG hydrogel with gelatin methacrylate (GelMA) hydrogel [81]. Another advantage of using PEG-GelMA hydrogel was that cells were able to attach on the hydrogel because of the existence of gelatin which is a natural protein. Since the cell attachment (also known as cell adhesion) has been proven to be the molecular basis of tissue architecture [82], it is important to have cell adhesive hydrogels for long-term cell culturing. Moreover, hydrogels as tissue scaffolds need to have tunable mechanical properties (i.e., stiffness and swelling ratio) that offer the effectively controllable microenvironment for cells to regulate their fate [83]. It has been reported that hybrid PEG-GelMA hydrogels have tunable mechanical properties [81], [84].

Therefore, this study developed the PEGDA-GelMA hybrid bioinks with the Eosin Y-based visible light photoinitiator to build scaffolds using the developed SLA-based bioprinting system. The comparison of two existing hydrogels and the proposed hydrogels are given in Figure 3.1 to clearly illustrate the difference and novelty of PEGDA-GelMA hybrid bioinks.
Table 3.1 Comparison between previously reported hydrogels and the developed hydrogels. (*: Determined by the experimental results presented in this chapter.)

<table>
<thead>
<tr>
<th>Sources</th>
<th>PEGDA</th>
<th>PEG-GelMA</th>
<th>PEGDA-GelMA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity of light band</td>
<td>Green light (~ 514 nm)</td>
<td>UV light (~ 365 nm)</td>
<td>Green light (~ 514 nm)</td>
</tr>
<tr>
<td>Controllable stiffness</td>
<td>No</td>
<td>Yes</td>
<td>Yes*</td>
</tr>
<tr>
<td>Cell adhesion</td>
<td>No</td>
<td>Yes</td>
<td>No*</td>
</tr>
<tr>
<td>Comments</td>
<td>One representative visible light crosslinkable hydrogel</td>
<td>One representative UV crosslinkable tunable hybrid hydrogel</td>
<td>First visible light crosslinkable tunable hybrid hydrogel</td>
</tr>
</tbody>
</table>

3.2 Crosslinking Mechanism and Optimization

This section aims to provide the introduction of the visible light crosslinking mechanism of Eosin Y-based photoinitiator. There are two ways of Eosin Y-based photoinitiation for hydrogels. First, the only Eosin Y was used as the photoinitiator [86], [87]. In this case, hydrogels had to be modified to have norbornene groups, which could be crosslinked by the thiy radicals generated by the Eosin Y after irradiation [86]. However, the synthesis process to add norbornene groups to common hydrogels (i.e. PEG) was complex and time-consuming, which limits the application of only Eosin Y crosslinking. Second, Eosin Y was used as the photosensitizer, TEA as the initiator, and NVP as the accelerator [75], [88]. Such multi reagents crosslinking method is suitable for crosslinking many types of hydrogels with acrylate groups and has been widely used for cell
encapsulation in tissue engineering. Therefore, we adopted the multi reagents crosslinking method for the Eosin Y-based photoinitiation.

The crosslinking mechanism of Eosin Y with multi reagents was firstly discussed by Valdes-Aguilera et al [89]. When irradiated by a green light, Eosin Y’s spectral properties in polar solvent change and make the Eosin Y to plunder reductive electrons from the electron donor, such as the TEA. After the electron transfer, TEA forms TEA radical cation (TEA⁺) and crosslinks the acrylates. Cruise et al. found that the involvement of NVP could significantly increase the crosslinking efficiency, as determined by the thickness of the hydrogel crosslinked at a certain time while the cell viability was not significantly decreased [88]. Bahney et al. further studied the optimized concentration of multi reagents based Eosin Y photoinitiation to crosslink stem cell-laden PEGDA hydrogels [75]. They reported that the minimum concentration of the three reagents offering acceptable crosslinking results was 0.01 mM Eosin Y, 0.1% w/v TEA, and 37 nM NVP. They also reported a typical absorption peak of the three reagents Eosin Y photoinitiation, as shown in Figure 3.1. It can be seen that the absorption peak of Eosin Y was around the wavelength of 510 nm, and most of the absorption happened from 450 to 550 nm. The fastest way to activate Eosin Y photoinitiator is to apply the strong and pure green light with the wavelength of 510 nm, which is impossible with the halogen lamp built in the beam projector. Therefore, in order to get the shortest crosslinking time, we used the white light, which covers the entire spectrum of visible lights, to crosslink Eosin Y photoinitiator-based bioinks.
3.3 Hydrogel Preparation

Table 3.2 Detail information of Eosin Y-based hybrid bioinks.

<table>
<thead>
<tr>
<th>Name</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PEGDA</td>
</tr>
<tr>
<td>10% w/v PEGDA</td>
<td>10% w/v</td>
</tr>
<tr>
<td>5% w/v PEGDA + 5% w/v GelMA</td>
<td>5% w/v</td>
</tr>
<tr>
<td>2.5% w/v PEGDA 7.5% w/v GelMA</td>
<td>2.5% w/v</td>
</tr>
</tbody>
</table>

Figure 3.1 Spectrophotometric absorption of Eosin Y (Adopted from [75]).
Three different types of hydrogels, 10% w/v PEGDA as a control, 5% w/v PEGDA + 5% w/v GelMA, 2.5% w/v PEGDA + 7.5% w/v PEGDA, were prepared with the fixed concentration of Eosin Y-based photoinitiator as listed in Table 3.2 [85]. GelMA was synthesized by the process previously reported by [90]. Briefly, 5 g gelatin was dissolved in 50 mL dimethyl sulfoxide solvent at 50 °C with stirring. Then, 0.3 g 4-dimethylaminopyridine and 2 mL glycidyl methacrylate were added to the mixture and dissolved. The mixture was stirred for two days at 50 °C. The mixture was the dialyzed with reverse osmosis water at room temperature for five days. The water was changed twice a day. After dialysis, the solutions were freeze-dried via lyophilization for a week. Then, 10% w/v GelMA solution was achieved by dissolving freeze dried GelMA in PBS with Eosin Y-based photoinitiator. Finally, the PEGDA-GelMA hydrogels with different concentration were made by mixing 10% GelMA and 10% PEGDA together. All the materials were purchased from Sigma-Aldrich, St. Louis, MO, USA.

3.4 Characterization of Bioink Properties

To characterize the mechanical properties of the hydrogels, 5 mL of each hydrogel pre-polymer solution were pipetted into a petri dish (6 cm in diameter) and exposed to the developed visible light system for 12 minutes to achieve crosslinked hydrogels. Five cylindrical specimens (12.7 mm in diameter) from each type of PEGDA-GelMA hydrogels were cut from the petri dish using a punch. The compressive Young’s modulus of each sample was tested by a dynamic mechanical analyzer (DMA, Q800, TA Instruments, New Castle, DE, USA). The compressive modulus was calculated from the slope of the linear region between strains from 5% to 15% as shown in Figure 3.2a. One-way analysis of variance (ANOVA analysis) function in MATLAB 2014b (Mathworks,
Natick, MA, USA) was used to statistically analyze the data obtained from the experiments. Results are shown in the form of mean ± standard deviation.

The results of measured Young’s modulus are given in Figure 3.2b. The 10% PEGDA hydrogel had the highest compressive Young’s modulus (~200 kPa). After mixing it with GelMA, the Young’s modulus of the hydrogels was decreased significantly, because the GelMA is a relatively ‘soft’ hydrogel [90]. The Young’s modulus of 5% PEGDA + 5% GelMA and 2.5% PEGDA + 7.5% GelMA were 60 kPa and 20 kPa, respectively. Therefore, by controlling the ratio

![Figure 3.2](image_url)

**Figure 3.2 Young’s modulus measurement.** (a) Representative strain-stress curve. (b) Calculated compressive Young’s modulus. (*p < 0.0001, n = 5, adopted from [51])
between PEGDA and GelMA, we can easily tune the mechanical properties of the hybrid hydrogels. The tunability of the mechanical properties, as mentioned above, offers a great possibility to control the cellular microenvironment for regulating stem cell fate [91]–[93].

For the mass swelling ratio test, six cylindrical specimens were prepared using the same method as described above. The residual liquid of the samples was removed with a tissue paper. Then, the weight of the swollen sample was measured with a precision balance (Sartorius, Mississauga, ON, Canada).

Subsequently, these samples were lyophilized for five days to determine the weight of the dry samples. The mass swelling ratio was calculated by the following formula:

\[
\text{mass swelling ratio} = \frac{\text{the swollen weight of the sample}}{\text{the dry weight of the sample}}
\]  

(3.1)

![Figure 3.3 Mass swelling ratio measurement. (*p < 0.05, **p < 0.001, n = 6, adopted from [51])](image)
The swelling ratio of the hydrogels is an essential parameter of tissue engineering since it affects various aspects including surface properties, mobility and solute diffusion [94]. The swelling degree of hydrogels depends on the pore size of the polymer and the interaction between solvent and polymer [95]. The calculated mass swelling ratios of PEGDA-GelMA hydrogels are shown in Figure 3.3. The higher GelMA concentration was found to increase the swelling ratio. However, compared to the 10 w/v % GelMA hydrogel reported in [90], the average swelling ratios of hybrid hydrogels were significantly lower. The possible reason could be the low molecular weight of PEGDA that was used in this study.

Figure 3.4 Microstructure observation. (a) 10% PEGDA (Scale bar = 20 μm) (b) 5% PEGDA + 5% GelMA (c) 2.5% PEGDA + 7.5% GelMA. Scale bar = 100 μm. (Adopted from [51])
The microstructures of the samples were also examined by a SEM (Mira3 XMU, TESCAN, Brno, Czech Republic) with 10 nm thick gold – palladium coating. Figure 3.4 shows the SEM images of the hydrogel microstructures. The microstructure of 10% PEGDA was very dense as shown in Figure 3.4a. No pore with the diameter larger than 10 μm was observed. The dense structure explains higher compressive Young’s modulus of 10% PEGDA. At the same time, it could cause cell death during long-term culturing because it would be hard for the biomolecules in cell media to diffuse into the hydrogel. On the contrary, the microstructures of 5% PEGDA + 5% GelMA and 2.5% PEGDA and 7.5% GelMA contained many pores with diameters between 50 and 100μm (Figure 3.4b and c). Although large pore size results in the decrease of mechanical properties, it creates better microenvironments for cells to live and grow. The large pore size has been reported to help cells spread and proliferate [80], [96]. The microenvironment of the hybrid PEGDA-GelMA bioinks will improve the cell viability for the long-term culturing.

### 3.5 Bioprinting of Hybrid Bioinks

With the significant difference of microstructures between PEGDA and hybrid PEGDA-GelMA bioinks, the hybrid bioinks may improve cell viability for several days’ culturing of bioprinted samples. For bioprinting experiments, NIH 3T3 fibroblasts were cultured at 37 °C and 5% CO₂. The cell medium consisted of Dulbecco’s modified Eagle medium (DMEM) with 10% v/v fetal bovine serum and 1% v/v penicillin-streptomycin. The cell medium was changed every two days. All the materials used in this paragraph were purchased from Life Technologies, Grand Island, NY, USA.

To print cell-encapsulated mesh patterns, cells (5 x 10⁶ cells/mL) were mixed well with the PEGDA-GelMA hybrid hydrogel pre-polymer solution with the Eosin Y-based photoinitiator.
μL (approximately equal to 100 μm height in the petri dish) of the PEGDA-GelMA pre-polymer solution mixed with cells were evenly added to a petri dish (6 cm in diameter) using a syringe pump before crosslinking each layer. Before printing, the petri dish was treated by the oxygen plasma to make the surface of the petri dish hydrophilic for spreading the hydrogel pre-polymer solution evenly. 4-by-4 mesh patterns with 3 layers in total were printed. The crosslinking time of each layer for 10% PEGDA, 5% PEGDA + 5% GelMA, and 2.5% PEGDA + 7.5% GelMA were 6 minutes, 12 minutes, and 24 minutes, respectively. The more the GelMA concentration, the more crosslinking time were required since GelMA pre-polymer solution with Eosin Y-based photoinitiator needed more time to be crosslinked than PEGDA.

After the printing, the uncrosslinked hydrogel pre-polymer residue was removed by pipetting manually. Fresh media were added to the petri dish, followed by washing the printed sample with PBS three times. The samples were cultured for 5 days to check cell viability. Cell viability was examined at day 0 (right after the printing) and day 5 using the live/dead cell viability assay protocol described in section 2.4. The assayed samples were washed twice with PBS and examined under a confocal fluorescence microscope (FV1000, Olympus, Tokyo, Japan). With a 10 X objective, two fluorescent channels and one phase contrast channel were used to capture the microscope images. To avoid the crosstalk between laser signals, the sequential imaging mode was used to take a series of confocal fluorescence images with a 20 μm step in the Z direction. Fluoview ASW software (version 3.1a, Olympus, Tokyo, Japan) was used to stack the fluorescence and phase contrast images together. To analyze the cell viability, the images obtained from the microscope were converted to 16-bit gray value format, and the cell number was counted briefly with the particle counting function (Otsu method) in ImageJ software (NIH, Bethesda, MD, USA). Finally, the cell viability was calculated by the following formula:
Cell viability = \frac{\text{number of live cells}}{\text{number of all cells}} \quad (3.2)

One-way analysis of variance (ANOVA analysis) function in MATLAB 2014b (Mathworks, Natick, MA, USA) was used to statistically analyze the viability data obtained. Results are shown in the form of mean ± standard deviation.

The representative microscope images of 10% PEGDA and 5% PEGDA + 5% GelMA are given in Figure 3.5. From the images, most of the cells in both hydrogels were alive immediately after printing, and the cells were distributed evenly inside the pattern. Also, the patterns were

Figure 3.5 Representative microscope images of 10% PEGDA and 5% PEGDA + 5% GelMA. Scale bar = 200 μm.
clearly printed. Unlike the 10% PEGDA, 5% PEGDA + 5% GelMA hydrogel did not have any background noise from green dyes, which assumed that the residue of the green dye was washed away by the PBS after the staining. This phenomenon is due to that the permeability of the 5% PEGDA + 5% GelMA was much better than 10% PEGDA and biomolecules of green dye were washed away easily. On the contrary, at day 5, it was observed significant increment of dead cells in 10% PEGDA pattern, which matches with the previous results presented in Figure 2.11.

To quantitatively analyze the cell viability, the numbers of dead and live cells were counted using the method described previously. Figure 3.6 shows the viability of cells encapsulated in various hydrogels. It can be concluded that the viabilities of all three hydrogels at day 0 were similar, at around 85%. Although not statistically significant, the viability of 10% PEGDA is a little bit higher than the hybrid hydrogels. This phenomenon may be explained by the relatively shorter printing time. At day 5, as observed before, the viability of 10% PEGDA was decreased to less than 60%. As discussed in Section 2.4, the pore size of 10% PEGDA was too small to diffuse medium into cell inside the hydrogel.

Using higher molecular weight PEGDA may improve the cell viability, since higher molecular weight PEGDA can form a relatively loose network with larger pore size if the crosslinking time remains the same [79]. The larger pore size can benefit the exchange of biomolecules and thus promote long-term cell viability [97]. The small pore size problem has been greatly improved by mixing GelMA with low molecular weight PEGDA. The cell viabilities of hybrid hydrogels were largely unchanged between day 0 and day 5, revealing that the long-term biocompatibility of PEGDA-GelMA hydrogels is suitable as bioinks. Considering the difficulties of handling soft 2.5% PEGDA + 7.5% GelMA hydrogels, among the three types of hydrogels tested in the study, 5% PEGDA + 5% GelMA with Eosin Y-based photoinitiator is the best bioink.
solution for the developed visible light SLA-based bioprinting system. It offers not only long-term biocompatibility, but also relatively strong mechanical properties.

However, there is still a limitation of the 5% PEGDA + 5% GelMA hybrid hydrogels. The hybrid hydrogels crosslinked by the Eosin Y-based photoinitiator do not allow cell adhesion. As shown in Figure 3.5. None of the cells were attached on the hydrogels at day 5, which was the unexpected phenomenon. Hutson et al. reported that the hybrid hydrogel crosslinked by UV light allowed cells to attach to the surface of the hybrid hydrogel [81]. Since the cell adhesion capability of hydrogels is one of the important factors for regenerating proper tissue functions [82], the printed bioinks are highly desired to be cell attachable. With this demand, the developed PEGDA-GelMA hybrid bioinks are required to be further investigated for the bioprinting applications, which aim to regenerate artificial tissues and organs in vitro.

Figure 3.6 Cell viability analysis (*p < 0.05, n = 10) (Adopted from [51])
3.6 Chapter Summary

The PEGDA 700 Da hydrogel used in Chapter 2 had poor cell viability of culturing cells for 5 days. The main reason due to the low cell viability was the small pore size of the crosslinked PEGDA. In this chapter, GelMA hydrogel was synthesized and mixed with PEGDA hydrogel to develop a new hybrid visible light crosslinkable bioink with offering better cell viability. The mechanical and biological properties of the hydrogel were characterized systematically. The results revealed that the hybrid PEGDA-GelMA hydrogel contained much bigger pore sizes, which further provided high cell viability for 5 days’ cell culturing. Also, the stiffness of PEGDA-GelMA hydrogel was tunable in a wide range by changing the concentration of GelMA. The tunable mechanical properties will benefit different tissue engineering applications. The developed hydrogels were used to conduct 3D bioprinting experiments. Using the visible light SLA-based bioprinting system, cells were successfully printed and encapsulated in a mesh pattern, demonstrating the compatibility of the PEGDA-GelMA hydrogel with 3D bioprinting. The cell viability of PEGDA-GelMA hydrogel at day 5 was around 85% which verified the feasibility of the hybrid bioink for long-term culturing inside the printed samples.
Chapter 4: Visible Light Crosslinkable Cell-adhesive Bioinks

For tissue regeneration, it is important that cells attach inside the hydrogel network and proliferate to form a 3D ECM network. The hybrid PEGDA-GelMA bioinks presented in the previous chapter was not able to provide cell attachment. As discussed in Chapter 1, there are two types of hydrogels, which are categorized by natural hydrogels and synthetic hydrogels [28]. Synthetic hydrogels are made from purely synthetic components. PEG-based hydrogels are one of the most widely used synthetic hydrogels since PEG is one of a few hydrogels approved by the Food and Drug Administration (FDA) for biomedical and drug applications [98], [99] and offers a tunable degradation rate [100] and controllable stiffness [101]. However, PEG for tissue engineering applications is limited due to the non-adhesive nature for cells. Cell adhesion can be improved by conjugating PEG with recombinant proteins [102] or modifying the PEG with adhesive peptides [103]. However, such methods are complicated, and the improvement of adhesion is still limited. On the contrary, natural hydrogels are derived from natural proteins or polysaccharides [28]. Most of the protein-based hydrogels, including gelatin, collagen, and fibrin [104], offers an excellent surface for cell adhesion. The derivatives of protein-based hydrogels, such as GelMA, also has the cell adhesive surface [90]. Among all protein-based hydrogels, GelMA is the most cost-effective solution for tissue engineering applications [105] and has been used in many biomedical applications, including DNA biosensor [106], 3D cell culturing [107] and vascularization [108], [109].

4.1 GelMA Preparation for Visible Light SLA-based 3D Bioprinting

GelMA hydrogels can be crosslinked by various photoinitiators, such as Irgacure 2959 [90], and VA-086 [110], [111] and are suitable for 3D cell culturing for tissue engineering applications. As
discuss in Section 3.2, Eosin Y-based photoinitiator can crosslink acrylate groups [88].

Methacrylates are specific kinds of acrylates which have an extra methyl group attached to one side of the acrylates as shown in Figure 4.1. During the synthesis process, gelatin containing primary amine groups was reacted with methacrylic anhydride and thus GelMA possessed methacrylate pendant groups after synthesis (Figure 4.2a) [90]. Therefore, Eosin Y-based photoinitiator can be used to crosslink GelMA. With the presence of an appropriate concentration of Eosin Y, TEA, and NVP and the green light irradiation, GelMA can be crosslinked to form a hydrogel network (Figure 4.2b). In this study, various concentrations of GelMA and Eosin Y-based photoinitiator were examined to find the best combination of them for 3D bioprinting applications.

Figure 4.1 The general chemical formula of (a) acrylate and (b) methacrylate. Methacrylate is a specific acrylate with extra methyl group attached to one side of the acrylate. (R stands for any group of atoms, and H appeared in the formula can be replaced by CH₃)
GelMA hydrogel was synthesized using the same protocol described in Section 3.3. To test the minimum concentration of Eosin Y for successful crosslinking, various concentrations of Eosin Y-based photoinitiators and GelMA were used to form hydrogel scaffolds under the visible light, as shown in Table 4.1. The 1X concentration of the Eosin Y photoinitiators was based on the minimum concentration of Eosin Y used for crosslinking the hybrid PEGDA-GelMA hydrogel.
Table 4.1 Detail information of Eosin Y-based GelMA solutions.

<table>
<thead>
<tr>
<th>Name</th>
<th>Concentration</th>
<th>Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GelMA</td>
<td>Eosin Y</td>
</tr>
<tr>
<td>10% w/v GelMA with 1X concentrated Eosin Y initiator</td>
<td>10% w/v 0.01 mM 0.1% w/v 37 nM</td>
<td>1X 10% GelMA</td>
</tr>
<tr>
<td>10% w/v GelMA with 2X concentrated Eosin Y initiator</td>
<td>10% w/v 0.02 mM 0.2% w/v 74 nM</td>
<td>2X 10% GelMA</td>
</tr>
<tr>
<td>10% w/v GelMA with 3X concentrated Eosin Y initiator</td>
<td>10% w/v 0.03 mM 0.3% w/v 111 nM</td>
<td>3X 10% GelMA</td>
</tr>
<tr>
<td>10% w/v GelMA with 4X concentrated Eosin Y initiator</td>
<td>10% w/v 0.04 mM 0.4% w/v 148 nM</td>
<td>4X 10% GelMA</td>
</tr>
<tr>
<td>15% w/v GelMA with 1X concentrated Eosin Y initiator</td>
<td>15% w/v 0.01 mM 0.1% w/v 37 nM</td>
<td>1X 15% GelMA</td>
</tr>
<tr>
<td>15% w/v GelMA with 2X concentrated Eosin Y initiator</td>
<td>15% w/v 0.02 mM 0.2% w/v 74 nM</td>
<td>2X 15% GelMA</td>
</tr>
<tr>
<td>20% w/v GelMA with 1X concentrated Eosin Y initiator</td>
<td>20% w/v 0.01 mM 0.1% w/v 37 nM</td>
<td>1X 20% GelMA</td>
</tr>
</tbody>
</table>

4.2 Experiments for Crosslinkability Test

Table 4.2 Crosslinkability of GelMA with Eosin Y-based photoinitiator.

<table>
<thead>
<tr>
<th></th>
<th>1X Eosin Y</th>
<th>2X Eosin Y</th>
<th>4X Eosin Y</th>
</tr>
</thead>
<tbody>
<tr>
<td>10% w/v GelMA</td>
<td>N</td>
<td>N</td>
<td>Y</td>
</tr>
<tr>
<td>15% w/v GelMA</td>
<td>N</td>
<td>Y</td>
<td>N/A</td>
</tr>
<tr>
<td>20% w/v GelMA</td>
<td>Y</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

N: Not crosslinked, Y: Crosslinked, N/A: Not applicable
To test the crosslinkability of the various GelMA pre-polymer solutions listed in Table 4.1, 100 \( \mu \text{L} \) of GelMA pre-polymer solution was pipetted into a petri dish (6 cm in diameter) and irradiated the visible light for 20 minutes. The hydrogels were visually examined to determine whether they were crosslinked or not. The results are reported in Table 4.2.

It was found that the lower percentage of GelMA required the higher concentration of Eosin Y for crosslinking. The threshold values of Eosin Y concentration for crosslinking 10%, 15%, and 20% GelMA pre-polymer solution was 4X, 2X, and 1X, respectively. As reported by Bahney et al. [75], the cell viability was decreased with higher concentration of Eosin Y photoinitiator. Hence, three combinations of the threshold value (4X Eosin Y - 10% GelMA, 2X Eosin Y - 15% GelMA, and 1X Eosin Y - 20% GelMA) were chosen for the mechanical and biological studies to investigate the properties of different concentrations of GelMA crosslinked by the minimized amount of Eosin Y.

4.3 Characterization of Bioink Properties

Young’s modulus of the three hydrogels crosslinked by the visible light for 20 minutes was characterized by DMA machine using the protocol described in Section 3.3. Compressive Young’s modulus was determined by the slope of the linear region between strains of 5% and 20% from the stress-strain curves as shown in Figure 4.3a. With the increasing concentration of GelMA, the compressive Young’s modulus was improved for even the hydrogel crosslinked by the less concentration of Eosin Y (Figure. 4.3b). However, comparing with the 5% PEGDA + 5% GelMA, Young’s modulus of 20% GelMA was 14.0 \( \pm \) 2.32 kPa, which is 4 times lower than the one of 5% PEGDA + 5% GelMA. It is reasonable since GelMA is a relatively soft hydrogel as described in Nichol et al. [90]. GelMA with Eosin Y photoinitiator crosslinked by the visible light was softer.
than the one crosslinked by UV light [90]. One-way ANOVA was used to analyze the data. Results are shown in the form of mean ± standard deviation.

The mass swelling ratios were also determined using the protocol described in Section 3.3. As shown in Figure 4.4. The swelling ratio of Eosin Y crosslinked GelMA is not changed too
much, compared to the UV crosslinked GelMA [90]. 10% GelMA showed that the swelling ratios were around 7 – 8 and 15% GelMA was around 5 – 6.

The microstructures of the GelMA were investigated using the SEM. As shown in Figure 4.5, it was found that the pore size of hydrogels depended on the concentration of Eosin Y rather than the concentration of GelMA. 4X 10% GelMA had the smallest pore size while 1X 20% GelMA has the biggest one. In general, the pore sizes of GelMA crosslinked by Eosin Y photoinitiator were between 30 and 120 μm, which were greater than the size of single cells and suitable for the cell microenvironments. On the contrary, the hybrid hydrogel of 5% PEGDA + 5% GelMA with a general pore size around 50 μm did not provide the cell attachment. Therefore, cell encapsulation experiments would verify the difference between GelMA and GelMA-PEGDA hybrid bioinks in terms of the cell attachment.

Figure 4.4 Mass swelling ratio measurement. (*p < 0.001, n = 5)
NIH 3T3 fibroblasts were cultured with the same protocol described in the previous chapter. Each type of GelMA pre-polymer solutions with Eosin Y was mixed with NIH 3T3 fibroblast (3 x 10^6 cells/mL) before encapsulation. 100 μL of each type of GelMA pre-polymer solution with cells was evenly pipetted into the petri dish to form a drop. The drop was exposed to the visible light for 20 minutes. Immediately after crosslinking, the samples were washed with PBS twice. Then,
the petri dish was filled with 3 mL of fresh media and placed in the incubator for culturing cells encapsulated in GelMA hydrogel scaffolds.

Cell viability at day 1 and cell proliferation at day 1, day 4, and 6 were examined. Crosslinked samples were washed two times with PBS and treated with the live/dead assay for 30 minutes. Subsequently, the assayed samples were observed under a confocal fluorescent microscope with a 10X objective. Two fluorescent channels were used to capture the microscope images. A series of confocal fluorescent images with the 20 µm step in Z-direction were obtained. Confocal image analysis software (Fluoview ASW 3.1a, Olympus, Tokyo, Japan) was used to stack the fluorescent images. To analyze cell viability at day 1, the images obtained by the microscope were converted to 16-bit gray value format, and the numbers of live cells and dead cells were counted through cell counting function. The cell viability was finally calculated in the same way illustrated in Section 3.4.

Cell coverage rate as an indicator of cell proliferation was also investigated between day 1 and day 6. The green channel of the images of live cells obtained by the microscope at day 1 and day 6 were converted to 16-bit gray value format and normalized through histogram equalization function (histeq) through the image processing toolkit provided by MATLAB 2014b (Mathworks, Natick, MA, USA). Then, a threshold of gray value was set to filter the pixel with the brightness lower than the threshold. The gray values of filtered pixels were set to 0 (darkest black). Through the threshold filtering, only the pixels with high brightness (high green fluorescent signal, or equivalently, the pixels inside the cells) remains. Then the cell coverage rate was calculated by the following formula:

\[
\text{Cell coverage rate} = \frac{\text{Number of high brightness pixels}}{\text{Number of all pixels}}
\]  

(4.1)
The representative images of live/dead assayed GelMA hydrogels are given in Figure 4.6. At day 1, the cell density inside the hydrogel was low, and there were more dead cells in 10% GelMA, which may be due to the high concentration of Eosin Y photoinitiator. After four days’ culturing, the cells started to attach and elongate on the surface of the hydrogel. At day 6, most of the cells were attached and spread out, and the cell density was increased significantly compared to day 1. It was also noted that 20% GelMA allowed better cell attachment and proliferation. One way ANOVA was used to statistically analyze cell viability and coverage rate. As shown in Figure

Figure 4.6 Representative fluorescence images of cell-laden GelMA hydrogels at day 1, day 4, and day 6. Scale bar = 100 μm.
the lower concentration of Eosin Y photoinitiator contributed to the higher cell viability after crosslinking. Moreover, 1X 20% GelMA hydrogels offer better cell coverage rate than other two hydrogels. The possible reason may be the relatively large pore sizes and higher stiffness. The characteristics of different GelMA bioinks were summarized in Table 4.3. From the results, this study demonstrated that GelMA bioinks with Eosin Y-based photoinitiator could be crosslinked by the visible light for bioprinting applications. Although their mechanical properties are limited, the surface condition of GelMA is superior to hybrid PEG-GelMA bioink for the cell attachment and proliferation, making it become a good candidate for artificial tissue regeneration in vitro.
Table 4.3 Characteristics of GelMA Bioinks with Eosin Y-based photoinitiator.

<table>
<thead>
<tr>
<th></th>
<th>Stiffness</th>
<th>Cell Viability</th>
<th>Cell Proliferation</th>
<th>Viscosity</th>
</tr>
</thead>
<tbody>
<tr>
<td>4X 10% w/v GelMA</td>
<td>Low</td>
<td>Low</td>
<td>Low</td>
<td>Fair</td>
</tr>
<tr>
<td>2X 15% w/v GelMA</td>
<td>Fair</td>
<td>Fair</td>
<td>Fair</td>
<td>High</td>
</tr>
<tr>
<td>1X 20% w/v GelMA</td>
<td>Fair</td>
<td>High</td>
<td>High</td>
<td>Extremely High</td>
</tr>
</tbody>
</table>

For bioprinting experiments, the 2X 15% GelMA pre-polymer was selected and printed with cells because the 1X 20% GelMA was too viscous to handle and 4X 10% GelMA offered relatively bad cell attachment compared to 2X 15% GelMA. As shown in Figure 4.8, the cross junction
pattern was successfully printed, demonstrating the feasibility of the developed GelMA hydrogels for bioprinting applications. The live/dead assayed image at day 5 showed that cells were well attached and elongated inside the printed pattern after several day’s culturing.

4.5 Chapter Summary

Since PEGDA-GelMA hybrid hydrogels could not support cell attachment, the applications using the hybrid bioinks in tissue engineering were limited. In this chapter, GelMA, which is a derivative of natural hydrogels for better cell attachment, was used to test its crosslinkability through Eosin Y-based photoinitiation. The minimum concentration of Eosin Y for crosslinking GelMA was increased with the less concentration of GelMA. Comparing to the concentration of Eosin Y photoinitiator, the concentration of GelMA dominates the mechanical properties of the crosslinked hydrogels. In addition, with the less concentration of photoinitiator, the pore size of hydrogel microstructures was increased. Therefore, among three combinations of Eosin Y and GelMA, 1X Eosin Y – 20% GelMA provided the biggest pore size with the stiffest mechanical property. Cell encapsulation results indicated that the NIH-3T3 cells were able to attach on the GelMA hydrogel crosslinked by Eosin Y-based photoinitiation. The higher concentration of GelMA yielded the better cell coverage and proliferation. Although the mechanical properties of GelMA hydrogels are relatively soft, the developed visible light crosslinkable GelMA hydrogels is a promising candidate for the visible light SLA-based bioprinting in tissue engineering applications due to its capabilities of cell attachment and proliferation.
Chapter 5: Conclusion

5.1 Conclusion

Bioprinting is a promising solution for the generation of artificial organs. Among all bioprinting techniques, the stereolithography (SLA)-based bioprinting has the advantages in view of its cost, printing resolution, and printing speed. The SLA-based bioprinting system has become more and more popular in recent years. It has been employed to fabricate vascularized tissues, investigate cancer migration, culture stem cell in 3D microenvironments, and build *in vitro* drug screening platform. However, most of the existing SLA-based bioprinting systems are based on UV crosslinking mechanism, which has a high possibility to result in DNA damage and cell cancerization. Therefore, it is highly desired to have a visible light-based bioprinting system for tissue engineering applications.

In this thesis, a novel SLA-based bioprinting system utilizing visible light for crosslinking hydrogels was developed and characterized. The system is based on a low-cost conventional beam projector. The entire system costs less than CDN$ 2,000 and is able to achieve 9.6 cm x 4.8 cm printing area. The minimum printing resolution of the system was 50 μm. The crosslinking time of a 100 μm thick layer was around 2 to 4 minutes, depending on the type of hydrogels. The developed visible light crosslinking process was biocompatible and the cell viability right after printing (day 0) was around 85%, as investigated by the live/dead cell viability assay staining.

Two visible light crosslinkable bioinks, such as PEGDA-GelMA hybrid bioink and GelMA bioink, for the long-term culturing of bioprinted samples, were developed. PEGDA-GelMA hybrid bioink offer tunable mechanical stiffness and has the potentials to be used to fabricate various tissues. Though high cell viability encapsulated in the PEGDA-GelMA hybrid bioink was maintained up to 5 days, encapsulated cells in PEGDA-GelMA hybrid bioink were not attached
and elongated. Since the cell attachment is one of the important factors for regenerating tissues, further investigation was required to develop visible light sensitive cell attachable bioink. On the contrary, though GelMA hydrogel is relatively soft and has limitation to tune the mechanical properties. It provides a better cell attachment property since it is based on a natural ECM protein. According to the results of live/dead assayed images obtained from the confocal microscopy, most of the NIH 3T3 fibroblast cells were attached after four days’ culturing and their proliferation rate was increased up to day 6. Hence, GelMA bioink is a good candidate for the generation of artificial tissues. Taken together, there is no one-for-all solution for the development of bioinks. However, the PEGDA-GelMA hybrid hydrogels and GelMA hydrogels can be applicable to many areas of 3D bioprinting and tissue engineering.

In summary, the objectives of the thesis were achieved through the development and demonstration of the novel visible light SLA-based bioprinting system and two visible light crosslinkable bioinks with different characteristics for 3D bioprinting applications. The developed visible light SLA-based 3D bioprinting system and visible light crosslinkable bioinks will be able to provide a promising bioprinting solution for a variety of tissue engineering applications.

5.2 Future Work

3D bioprinting is a rapidly developing research area. There are many directions for the future research, including the development of light based novel bioprinting techniques, the integration of the hybrid bioprinting system, and the in vivo studies of bioprinted tissues.
5.2.1 Direct Laser Bioprinting

In addition to the SLA-based 3D bioprinting system, we recently employed a focused laser for bioprinting. The focused laser can provide a high-intensity light source with the size of the printed area of 20 μm in diameter. Therefore, by controlling the movement of the focused laser, the hydrogel pre-polymer solution will be able to be selectively crosslinked. A preliminary study has recently published in [112]. It was found that the focused laser could create a 400 μm in diameter

Figure 5.1 Laser crosslinked microtube sample. (a) A phase contrast image (b) Fluorescence live/dead assayed image. Scale bar = 200 μm. (c) 3D reconstructed image of the printed microtube. Scale bar = 215 μm, adopted from [84])
hydrogel microtube using a linear stage with the movement speed of 3 mm/s. Figure 5.1 shows the live/dead assayed images of cell-laden microtubes after three days’ culturing. The cell viability encapsulated in the GelMA hydrogel was higher than 90%. An on-going research is building a novel bioprinting system based on direct laser crosslinking. Currently, the system is able to print the complex patterns of 30 μm in diameter within 5 minutes. The direct laser bioprinting system may offer better resolution and be a complementary system for the developed visible light SLA-based 3D bioprinting system in this thesis.

5.2.2 Hybrid Bioprinter

Integrating various 3D bioprinting techniques to build more complex structures, which closely mimic human organs, has been recently emerged. A recent study combined the extrusion- and SLA-based printing system together to build a hybrid bioprinting system shown in Figure 5.2 [45]. Although the two sub-systems were not able to work at the same time, the combined system offered a rapid solution to fabricate complex heterogeneous tissues. For example, the SLA-based sub-system could fabricate the majority of tissues with the advantages of fast fabrication and high-resolution. At the same time, the extrusion-based sub-system printed vessel-like structures in the majority of tissues with the advantages of multi bioinks printing capability. Integrating two subsystems together will be the promising solution to overcome the shortcomings of the single system and realize to print human organs in the laboratory in the near future.
Currently, most of the bioprinting studies only examined the cell viability and cell functionality *in vitro*. Since the ultimate goal of 3D bioprinting is to use printed tissues for transplantation, more researches need to investigate the fusion and functionalization of printed tissues *in vivo*.

Kang *et al.* recently reported the *in vivo* study of the bioprinted samples [47]. They found that the bioprinted skeletal muscle tissues could undergo vascularization and nerve regeneration process *in vivo* to form a functional muscle tissue which was responded to the electrical stimulation. After four weeks’ maturing *in vivo* environment, the printed tissues were able to perform muscle-like tissues and generate the significant amount of forces when contracted.
Since many *in vitro* studies have already been conducted *in vivo* study using the bioprinted tissues and test their responses to the complex physiological environments will be a major step to further close to the tissue or organ translation. Once the feasibility of the bioprinted tissues *in vivo* is well-studied, 3D bioprinting will be used in real life and applied to many clinical trials.

5.3 My Contribution

This section provides a brief review of outcomes during my master studies. I have published eight journal articles as listed below: (* equal contribution)


I also co-authored two book chapters as listed below:


I gave or will give presentations as listed below:


Bibliography


with and without a gold transductive layer: a parametric study,” in *Optical Interactions with Tissue and Cells*, 2016, vol. 9706, p. 97060O.


Appendices

Appendix A

This appendix covers the detailed information for the STL slicer employed in Chapter 2. The visible light SLA-based bioprinting utilized a layer-by-layer printing method. Therefore, the conventional 3D model designed by the computer must be converted to a series of 2D patterns before printing through a slicing software (Figure S1a). The slicing software used in the thesis is Freesteel Z-level slicer, which is available to download from http://www.freesteel.co.uk/wpblog/slicer/. The slicer is based on command line control, which is not user-friendly. Segwick 3D (Kansas City, MO, USA) integrated the slicer to a 3D printing

![Figure S1. STL slicing process. (a) Schematic of the slicing process (b) Snapshot of the Flashpoint software offering a controllable and express slicing function.](image)

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software named Flashpoint. The Flashpoint is a free software and can be downloaded from http://sedgwick3d.com/FlashPoint/. This software provides a graphic interface for the slicing process. It also supports the preview of each layer before saving as a series of image files. The interface of Flashpoint is shown in Figure S1b. The interface can be divided into three parts: 1) Selection of layers (in red dash line), 2) Preview and adjustment of selected layer (in blue dash line), and 3) Printing of the sliced patterns (in green dash line). This free software offers an express way of slicing the 3D model. The height of each layer is also adjustable. All 3D models used in the thesis were sliced by Freesteel Z-level slicer through the graphic interface provided by Flashpoint. Sliced models were exported as groups of binary BMP image files for the projection.
Appendix B

This appendix describes the principle of live/dead assay and imaging. Cell viability is an essential component of *in vitro* cell culturing. It is also the key factors when determining the biocompatibility of specific materials or engineering processes. In general, culturing condition, experimental handling, and biofabrication techniques can affect the cell viability directly or indirectly. Live/dead staining based on fluorescence dyes is one of the widely used methods to investigate the cell viability.

The live/dead assay contains two types of fluorescent dyes such as Calcein acetoxyethyl (Calcein AM, green dye for live cells), and ethidium homodimer (EthD, red dye for dead cells). The specific staining of live or dead cells is based on the membrane permeability of cells. The cell membrane of the live cell is selectively permeable to specific ions and organic molecules. Once cells die, its membrane is collapsed because no more adenosine triphosphate is supplied. As a result, the cell becomes permeable to everything. Calcein AM is a membrane-permeable molecule. Upon entering the cell, intracellular esterase cleave the AM ester group, transferring membrane-permeable Calcein AM to membrane-impermeable Calcein. Therefore, after the washing step, only live cells retain the Calcein AM inside the cells. The Calcein AM stains the entire area of the live cells inside the membrane. At the same time, EthD is a group of membrane-impermeable molecule bonded to the DNA. Because of its impermeable nature, it also combines with the DNA of dead cells. Therefore, the EthD stains the nucleus of the dead cell. There are several sub-types of EthD. The EthD-III used in the thesis is an enhanced version of basic EthD-I, offering stronger fluorescence signal under the fluorescence microscope.

Both Calcein AM and EthD-I are fluorescent dyes. Therefore, under a specific condition, they generate detectable fluorescence signals, indicating that cells are alive or dead. Fluorescence
signals describe a phenomenon where a molecular system absorbs a short wavelength light and then emits a long wavelength light (Figure S2). In the absorption, the short wavelength light (with high energy) excites the molecule, promoting electrons within the molecule to transit from the ground state (State 1) to excite state (State 2). Just a few pico-seconds after the excitation, the excited electrons relax in the lower available energy state (State 3). After a lag period of several nano-seconds, the electrons relax back to the ground state, releasing stored energy in the form of the emitted photon. The energy difference between State 1 and State 3 is lower than State 1 and State 2. Therefore, the emitted light has lower energy (in other word, longer wavelength).

The absorption and emitting spectrums of Calcein AM and EthD-I are shown in Figure S2. The dye supplier, Biotium, does not provide the spectrum of EthD-III, but it indicates its spectrum is similar to EthD-I. The data were measured and provided by Thermo Fisher Scientific, Waltham, MA, USA. As can be seen from the figure, the absorption peaks of Calcein AM and EthD-I are around 490 nm and 520 nm, respectively. The emitting peaks of Calcein AM and EthD-I are around
510 nm, and 630 nm, respectively. During the confocal imaging, the laser having the highest absorption (high relative intensity in Figure S3) to excite the fluorescent dye was applied. Olympus Fluoview FV1000 confocal microscope supports four excitation wavelengths (405nm, 488nm, 559 nm, and 635 nm). 488 nm and 559 nm laser were used to excite Calcein AM and EthD-III.

Figure S3. Fluorescence spectrum of Calcein AM and EthD-I. (Dash line indicates the absorption and solid indicates the emitting). Copyright Thermo Fisher Scientific
Appendix C

This appendix describes the details regarding the cell counting using ImageJ, which is a public domain Java image processing program developed by the NIH (Bethesda, Maryland, USA). ImageJ has many built-in functions for image processing and supports various programmed plug-ins for specific applications. For cell viability calculation, a cell counting function using ImageJ with the packages of plug-ins provided by Wright Cell Imaging Facility (WCIF, University Health Network, Toronto, ON, Canada) was used. The modified version of ImageJ is called WCIF ImageJ and available for downloading at http://www.uhnres.utoronto.ca/facilities/wcip/.

The cell viability was calculated through the following process:

![Image of cell viability calculation using cell counting function of ImageJ. (Scale bar = 200 μm)](image-url)

Figure S4. Cell viability calculation using cell counting function of ImageJ. (Scale bar = 200 μm)
1. Export the fluorescence image of Calcein AM (green, live cells).
2. Open the WCIF ImageJ software.
3. Convert the image to 8-bit gray image by using ‘Image->Type->8-bit’.
4. Count the cell number automatically through ‘Plugins->Particle Analysis->Nucleus Counter’.
5. The software will respond counted images with the total number of cells.
6. Repeat the process from 1 to 5 for the images of EthD-III (red, dead cells).

Representative images of original, gray, and counted Calcein AM and EthD-III channels were given in Figure S4.

These images were obtained from the 2X Eosin Y - 15% GelMA hydrogel at day 1. Most of the cells (> 90%) can be counted correctly through automatic counting. Manual counting may be conducted based on the results of automatic counting to cover a few missing cells. WCIF ImageJ Figure S5. Manual counting of cell number. Green squares indicate the position of live cell determined manually after automatic counting.
provides a built-in manual counting function, which can be found through ‘Plugins->Particle Analysis->Cell counter’ (Figure S5). In the end, the cell viability of a specific field of view is given by the equation (3.2) as shown below:

\[
\text{Cell viability} = \frac{\text{number of live cells}}{\text{number of all cells}}
\]  

(3.2)