Chapter 1
Introduction

1.1 Research Background

Organ deficiency requires an incredible requirement for the improvement of new biological alternatives to be created. Tissue engineering has developed as an interesting strategy to address this issue. Tissue engineering is a combination of interdisciplinary field that applies the principles at the intersection of medicine, biology, and engineering such as biomaterials science, cell-material interactions, cell biology, and surface characterization. It aims to meet the demand for creating structurally and functionally of new tissue by restoring, preserving to enhance tissue functions [1]. The main technique for tissue engineering is to seed particular isolated cell from a biopsy onto a 3D scaffold. An extra construction method is needed to construct special architecture of tissue and organ interest that provide living cells at the same time [2]. As a viable option for cell-based treatments and tissue engineering, 3D bioprinting innovation has developed, supported by ongoing advances. 3D bioprinting offers access to place cell-laden hydrogel bioink in a layer-by-layer fashion, with the possibility for creating complex composite tissue constructs through high resolution geometric such as; volumetric structure of customizable shape, size, internal architecture of tissue and organ interest [3,4].

One of 3D bioprinting's major challenges is to create a printing material to fulfill a suitable printing. The selection of bioink is challenging in 3D bioprinting, which is need to meet some requirements that a ~~–e~~ell friendly condition", including: biocompatibility and controllable degradation rate; tunable mechanical properties; precise control of multi-scale internal architecture of scaffolds to represent diverse construction of natural extracellular matrix (ECM); macro-scale pores to facilitate cell attachment and growth, and micro-scale pores to facilitate growth factors release and nutrient transport to surrounding cells [5]. Hydrogels have great potential as printing materials because of their ability to encapsulate cells and their imitation behavior of ECM [6]. The problem is the delicate balance between hydrogels' printability and biological properties against 3D bioprinting. Increasing the concentration of

polymer results in a highly viscous hydrogel precursor and fast gelation into a crosslinked hydrogel, which gives great and high quality of printability [7,8], but thick polymer network will impede new ECM and matrix remodeling and cell migration [9]. Hence, the improvement of a hydrogel framework with sufficient printability balance and cell support would facilitate the application of hydrogel in 3D bioprinting. Natural polymers have a similar characteristic to human ECM, their biocompatible and bioactive properties are excellent. But their mechanical properties are weak. Whereas, synthetic polymers are more easily to tailor because they have stronger mechanical properties than natural polymers. According to previous studies, gelatin as a natural material has been widely utilized for tissue or organ regeneration. Gelatin, a hydrolyzed collagen, has attracted considerable attention. It has a greater antigenicity and a lower cost than collagen. Because of its normal sequences of RGD, which can promote biological growth and cell-cell contact for many different cell types [10]. Gelatin seems to be a promising tool for tissue engineering because of its good properties [11]. Nonetheless, a chemical modification of this polymer is necessary to improve its final physicochemical properties and to improve the thermo reversibility due to its relatively low melting point [12].

Gelatin, which is thermally sensitive, can support the printing process [13,14]. In addition, gelatin can be altered by methacrylamide and a minority of methacrylate group, resulting in a photo-crosslinkable material-gelatin methacryloyl (GelMA). GelMA preserve gelatin biofunctionality and its photocrosslinkable property allows for the rapid creation of a covalently crosslinked hydrogel that maintains the printed structure indefinitely and thus becomes stable under physiological temperature. Modification of gelatin with photocrosslinkable methacrylate groups, not only maintains the unique properties of gelatin, but also provides the material to be solidified permanently via undergoes photoinitiated radical polymerization to form covalently crosslinked hydrogel. High crosslinking degree of polymer can be achieved by the substitution degree of MA. Just only minutes or even seconds at low concentration of PI, to minimizing cytotoxicity. In addition, the transparent nature of this modification hydrogel allows for easy observation of cellular behavior encapsulated within as seeded onto the hydrogel [10].

For the methacrylation of gelatin molecules, amino functional groups are typically used to bind with methacrylate groups, such as gelatin-methacylate anhydrate. Nonetheless, in each gelatin molecule, the number of amino groups is reduced. Within gelatin molecules there are many classes of hydroxyl and carboxyl on glycidyl methacryloyl will react with them. The use of these modification functional groups for further methacrylation of gelatin is desirable in order to increase the density of gelatin hydrogel crosslinking [15].

1.2 Literature Review

1.2.1 Tissue Engineering

Tissues and organs are damaged by age, disease, or trauma affected the patient's quality of life. Alternatively, transplantation and tissue reconstruction may be used. Biocompatible materials are required to approach this challenge, rely on various implementations of conventional surgical techniques for reconstruction the loss and failure tissues such as allograft, autograft, xenograft tissues and prosthetic materials [16]. Physicians and scientists searching for an alternative medical procedure with aim to replacement or restoration of diseased or damage tissue function from patients through organ transplantation that contain specific population of living cells then designed and constructed to meet the needs of each individual patient by combining new devices and material sciences with cell biology [17]. Tissue engineering offers a new solution that provide a proper environment for cell to attach, growth and proliferate while maintaining strong construction for improving sophisticated structure of implantable grafts [18]. The main basic principles of regenerative tissue engineering are including isolate cell from healthy source, develop cell for expand and growth, seed into a matrix/carrier/scaffold (encapsulated cell) then finally insert the graft in a patient's body by surgery [19,20].

1.2.2 3D Bioprinting

3D printing has greatly facilitated design and development of medical product such as personalized prosthetic material of medical and care [21-24]. A three-dimensional printing can customize an ideal scaffold to facilitate localization, mimic the native tissue characteristics and distribution of cell to

specific site in the body, that allow for flow transport of nutrient and cell growth and metabolic waste by an optimal mechanical and physical properties [25]. A revolutionary era of 3D printing is a adaptation of 3D printing is an adaptation of 3D printing from a traditional manufacturing processes to advanced recent manufacturing processes. This technique used combination of biocompatible materials such as natural and synthetic materials as biomaterial ink. The goal of this developed technique can guide physical, chemical, and biological of the cellular environment for promote tissue regeneration by controlling of diversity and complex architectures of human tissues and organs to achieve tunable mechanical and structural properties for enhanced and customized cellular response [26]. One of the advances on medical science is the three-dimensional bioprinting. It has gained significant attention feasibility towards the synthesis of living cell and biomaterial. Known as bioprinting, this technology is using a computer-aided design model for simultaneously extruded a bioink through a deposition nozzle and layer-by-layer that continuously writing of living tissue or organ with precise layering to fabricate bioengineering constructs for regenerative medicine, tissue engineering or other biological studies [27,28].

The most important difference between tissue engineering 3D printing and 3D bioprinting is the type of raw materials they are using. Bioprinting involves printing live cells and other biological materials. The approach of 3D bioprinting can be divided into three stages: pre-processing, processing (actual printing) and post-processing [29]. Preprocessing is a reconstructed image or version of relevant tissue or organ in CAD using complex geometric data from medical imaging techniques which include MRI, X-ray imaging, and μ-CT-scan. The use of 3D bioprinting in the biomedical field has several benefits, including the production of high precision, customized patient-specific prototypes, low cost on-demand construction of complicated systems within a quick time [30-31]. Consequently, the actual printing is done through a bioprinter after the creation of the blueprint by simultaneous deposition of cells and biomaterials using computer-aided precision deposition techniques is a layer-by-layer fashion. As the last step, the incubation of printed tissue construct for tissue remodeling, cell proliferation, and maturation in a specially designed chamber –bioreactor" that accelerates tissue maturation.

Despite the great advantages of 3D bioprinting in tissue engineering, based on the state of the art, the printing process of complex tissues and organ for transplantation has several drawbacks. First of all, there are obstacles when the process of adjusting the position of cell seeding into the micro-architecture construction of the scaffold. The challenge is how to sow, place, and form construction patterns according to design with high precision. Nevertheless, in the natural tissues and organs, many cell type classes are organized and communicate in very complex patterns. Furthermore, cells with high density will experience difficulty during the process of penetration into the scaffold gap. Because it is only able to spread on the surface of the scaffold, and difficulty in breaking into the scaffold gaps. Often cell growth is not optimal because it does not get enough space on the scaffold. This has caused many researchers to innovate in developing 3D bioprinting technology, where cells can be encapsulated to protect cells from the environment that can be destroyed during the printing process but can maintain the appropriate characteristics can be adjusted according to a predetermined pattern. Whichever is anatomically, the cells that have been seeded will be able to interact and develop well then regenerate with organs and tissues native [32].

1.2.3 Bioink

A soft biomaterial filled with living cells and labeled "bioink" as a bioprintable material is used in bioprinting processes. In order to create complex biological structures, scientists should control and evolve biological and biochemical conditions as well as living cells. Bioink's indicators of achievement are cell support for cell survival during short- and long-term development, cell-cell and cell-ECM interaction, cell spread and proliferation, and the durability of bioprinted buildings [33]. Customizing bioink will provide the mechanical, physical, chemical, and biological properties of printing structure by development of tunable bioink methods. Biomaterial of bioink must complete the required ink characteristic, not only should be extrudable enough from a specific diameter, size or shape of nozzle, but also mechanically weak enough to be extruded, yet stiff enough to be self-supporting upon deposition. Bioink's solution must be either very viscous or quickly gelled on the

printing substrate. Nonetheless, high polymer fraction solutions (> 5 wt%) provide the required viscosity for the concept of printing but are not ideal for tissue engineering since dense polymer matrices can inhibit matrix remodeling and in vivo vascularization [34,35].

1.2.4 Hydrogel

Hydrogels are a crosslinked network of hydrophilic polymers capable of holding large amounts of water and becoming significantly larger than their original weight in aquatic medium without dissolving. This characteristic of hydrogel also referred to as biocompatibility of hydrogel, that ideal for cell encapsulation [36,37]. Hydrogel is one of the most viable substance of bioink materials, are formed from either natural hydrogel or synthetic hydrogels. Natural hydrogels can imitate the native tissue environment since they have many important characteristics of the native ECM components [38] by facilitating matrix remodeling, cell-cell adhesion and cell migration that necessary for normal development of a functional tissue [39]. There are certain drawbacks to the findings of both natural and synthetic hydrogels. Natural hydrogels are more biocompatible, but unfortunately have poor mechanical properties to imitate the native tissue microenvironment [40], whereas synthetic equivalents lack major components such as bioactive molecules for cell migration or adhesion [41].

1.2.5 Crosslinking Mechanisms of Hydrogels

Hydrogels are produced primarily through the cross-linking of stable polymer networks. There are two fundamental strategies among different methods of cross-linking; physical and chemical cross-linking.

1.2.5.1 Physical Crosslinking

One of the most common solutions to hydrogel stabilization is the physical activity of the crosslinking system of hydrogels. Physically related hydrogels are typically connected to weak forces such as hydrogen bonds, ionic interactions, and van der Waals forces are commonly referred to as reversible systems. There has been a growing interest in physically cross-linked hydrogels for

encapsulation of cells, proteins and drugs in recent years. As for the main reason, the integrity of each cell and biomolecules in hydrogen are not influenced by interconnected agents [42]. Chemical crosslinkers can produce undesirable chemical waste-products and become toxic if applied to the biomedical field. So that physical crosslinking is more popular in biomedical safety because it eliminates unwanted chemical reactions. Especially under room temperature, crosslinked hydrogels can stimulate self-healing properties so that they can function as bioactive materials in cell encapsulation and provide medicines for therapeutic molecules [43]. An environmental cause such as changes in polyelectrolyte charge density, crystallization, pH, protein interactions, temperature, and secondary interactions (hydrophobic, electrostatic, and hydrogen bonding) is used to perform physical crosslinking. Physically regulated hydrogel networks typically have low stability in *in vitro* and *in vivo* conditions and weak mechanical strength [42].

1.2.5.2 Chemical Crosslinking

Comparing with physical crosslinking, hydrogels that are chemically crosslink have covalent bonds between polymers with greater durability so that the mechanical properties become higher than physical crosslinking. The advantages of chemical crosslinking are the characteristics of shear, tensile, bending, etc. [42-44]. Most of the covalent bonds in crosslinking chemically usually formed between polymer chains have strong and permanent properties. This improves consistency in physiological conditions and mechanical properties as well as controllable degradation characteristics. This is also an advantage when compared to physical crosslinking [45]. The crosslinker concentration used in the hydrogel cross-linking process can influence the degree of crosslinking. Hydrogel's excellent mechanical properties benefit from a high degree of crosslinking. Nevertheless, this function may minimize the hydrogel's degradation time, growth factors, drug release, or other biologics that are immobilized to spread into the surrounding tissue area. Because of stronger encapsulation between polymer chains that are chemically linked [42].

Based on the advantages of this photo-activated crosslinking process, the short process of forming the hydrogel network, the use of low photo-activated temperatures, and the regulation of easily adjustable cross-linking reaction arrangements allows to improve the mechanical properties of the hydrogel. The crosslinked site can be precisely selected under light exposure. Since photo-initiated polymerization requires only irradiating areas in the crosslinking of hydrogel [45]. In this procedure, UV light causes the initiation of polymerization of photoinitiator molecules through hydrogel solutions. It usually takes a few seconds to several minutes for the crosslinking process. To ensure high cell viability and reproducibility, reaction parameters such as concentration of the photoinitiator, UV exposure time, UV light intensity, and also unreacted monomers and radicals can damage cells and cell components, so the distance between the hydrogel specimen and the UV light source must be precisely controlled [42]. Irgacure (2-hydroxy-1-[4-(2-hydroxyethoxy)phenyl1]-2-methyl-1-propanone) is widely used as a photoinitiator in bioprinting [46]. Due to the relatively low cytotoxicity of UV-induced free radicals, Irgacure 2959 at concentrations of 0.05−0.5 w/v% were the most effective visible light initiating systems [45].

Nonetheless, there are still questions about DNA disruption, unwanted crosslinking, or UV-caused cell functionality. Water solubility, type of radiation and exposure time required to produce free radicals are therefore important criteria to be considered [47].

1.2.6 Gelatin

Gelatin with its biocompatability and biodegradability properties in the physiological environment because it is a natural polymer that still retains the unique properties of collagen, therefore it is broadly used in medical and pharmaceutical applications [48-49]. In fact, gelatin antigenicity is relatively lower than collagen. In addition, gelatin preserves the peptide sequence of RGD that promotes certain cell behaviors (such as adhesion, proliferation and differentiation) and a sequence of degradation of the MMP which promotes cell enzyme degradation [50-51].

Nonetheless, gelatin's thermostability is low (gelatin becomes a solution when the temperature is above 37°C due to cleavage of hydrogen bonds), and chemical crosslinking may affect gelatin's biocompatibility as some crosslinking reagents are toxic. Consequently, the application of gelation is constrained by the insufficient thermostability and possible toxicity of chemical crosslinking. Fortunately, gelatin side chains have large active groups, like –OH, –COOH, $–NH_2$, –SH, etc. Therefore, gelatin can be adjusted with different groups in order to overcome these limitations.

1.2.7 Gelatin Methacryloyl (GelMA)

GelMA is a gelatin derivative that includes a majority of methacrylamide groups and a minority of methacrylate groups. Tamayol and friends, propose that "gelatin methacryloyl" is a more appropriate name, which also refers to the broadly accepted GelMA abbreviation [52]. The method of synthesizing a hydrogel by adding methacrylate groups to the amine-containing gelatine side groups has been commonly used in biomedical applications because of its simple and controllable manufacturing [11]. GelMA includes gelatin as its backbone, and offers cell-responsive features such as the provision of suitable cell adhesion sites and proteolytic degradability [53]. Since it was first described by Bulcke [50,54], GelMA has proven to be one of the versatile hydrogels in cell laden-based 3D bioprinting applications. Modified hydrogel by gelatin and methacrylamide groups are semi-synthetic hydrogels that support the attachment of biological signals in gelatin molecules that can be modified so that it is possible to improve their mechanical properties [55]. Hydrogels obtained from gelatin derivatives with methacrylate anhydride still maintain the supporting biomaterial properties, such as thermoreversible physical binding which can maintain tendency of biological characteristics. This is the result of derivatization, which results in a modification of the hydroxyl and lysine residues with the methacrylate and methacrylamide side groups. Consequently, the GelMA hydrogel may facilitate the adhesion, development and proliferation of cells with a aqueous environment [52].

1.2.8 Gelatin Glycidyl Methacryloyl (GelGMA)

Gelatin chains carry different functional groups along the chain, including carboxylic, hydroxy and amino groups. The number and variety of these reactive sites open up a wide range of potential reactions. Glycidyl methacryloyl has been used with C=C double bonds to functionalize the reactive side groups of the peptide backbone chain. The gelatin thus becomes crosslinkable, enabling the development of new materials with quite unusual properties [56]. Glycidyl methacryloyl is a methacrylic acid ester from an epoxy group having a good long-term mechanical disintegration capability of hydrogel so that it is one of the popular crosslinkers used. With epoxy property offered by gycidyl methacryloyl, which provides polyolefins and other acrylic polymers with epoxy properties. The good thermal properties of this blend are able to form hydrogels more easily compared to methacrylate-based polymers. As a consequence, influenced polymer's inner morphology, different surface areas of the pore, and distributions of pore size. In addition, it can efficiently combine with monomers to create homogeneous solutions for the synthesis of hydrogels by copolymerizing free-radical solutions [57]. The association between polymerization temperature and porous structure is a result of the initiator's increasing rate of decomposition to increase temperature [58].

Glycidyl ethers are a fascinating and diverse class of monomers as it is possible to obtain a great variety of polymeric materials by changing the structure of the ether side group attached to the epoxide. GMA is a monomer of special interest as it has two polymerizable functions: epoxy and methacrylate. A major concern is to find conditions of polymerization that allow one of the functional groups to react selectively, thus preventing cross-linking reactions and producing unknown materials. Literature data on GMA polymerization almost exclusively reports reaction mechanisms involving methacrylate function more or less selectively while the epoxy ring remains unreacted [59].

1.3 Research Motivation and Objectives

Tissue engineering provides an alternative approach to meet the increasing need for organ transplants. There is a gap between the number of patients that can be taken while waiting for transplants and the number of donors available.

The objective of tissue engineering is to create functional organs from the cells of the patient [60]. However, this method is not an easy task. Although, human body has the ability to regenerate cell that limited by various factors such as the need for differentiation with growth hormones, tissue's type, and physical size and shape (critical defect). Any tissue injury that exceeds this critical size must be supported externally. The external support is known as scaffolding. Such scaffolds provide a platform for the cell to move to the active site and create new tissue [60]. There are several ways to design 3D bioprinting scaffold, this process can be achieved with several benefits including the ability of 3D bioprinting to create scaffold with complex construction and geometry, the use of biomaterial materials and components capable of making tissues that facilitate various cell types to adapt and the material is easily degraded to support the process of cell regeneration [61].

The bioprinting principle that the biomaterial is printed layer by layer method in the form of liquid until the entire object is manufactured. The biomaterial is solidified to retain the shape immediately after the liquid-shaped biomaterial leaves the print head. Converting this cycle from sol to gel or phase transfer [60]. The ink extrusion process which is repeated layer by layer directly which is stable and fast after the deposition process to create the design of biological structures (cells, tissues, organisms) as desired is the right expression for "3D biomaterials printing". Biomaterial inks usually contain living cells such as cell encapsulating ink or bioink, or those that do not contain cells such as acellular ink [35]. Biomaterial inks must satisfy conventional biomaterial requirements in terms of biocompatibility, biodegradability and must also be printable at the appropriate scales and architectures. Hydrogels is printed through a number of mechanisms: cross-linking layer by layer and as thickened solutions [35]. Hydrogel inks were printed with good cell stability, but when unmodified, they are' blank-slate' materials that missing cell degradation and cell adhesion locations. Such materials will allow certain sites to be inserted by adding sequences such as RGD and MMP-sensitive sequences to instill limited bioactivity, much less sufficient bioactivity to achieve appropriate tissue function and cellular [35].

Gelatin is typically modified for the preparation of gelatin-hydrogel with methacrylate groups. The limitation of this type of hydrogel for tissue engineering, especially for the tissue that requires extensive load-bearing properties and hydrogel degradation, is related to the crosslinking density of the hydrogel network [62]. The physical characteristics of hydrogel include swelling and degradation in particular. Swelling represent as hydrogel mechanical properties significantly affect the functional of the fabricated tissue constructs. Therefore, it is important to be aware of optimal hydrogel degradation to ensure that tissue growth is adequate in time in order to avoid the adverse effects of the hydrogel material around [62]. Amino functional groups are generally used to react with methacrylate groups for the methacrylation of gelatin molecules. Nonetheless, in each gelatin molecule, the number of amino functional groups is reduced. In gelatin molecules there are many groups of hydroxyl and carboxyl. Therefore, it is important to use these functional groups for potential gelatin methacrylation to increase the crosslinking capacity of gelatin hydrogel [63].

GelMA should have good printability as a bioink printer. Increasing GelMA concentration is one of the easiest approaches that can improve printability. High concentration, however, will result in highly interconnected networks within the hydrogel, which will restrict nutrient absorption or byproduct exchange [64], migration and cell proliferation [65], thus negatively influencing subsequent biological behaviors such as secretion and differentiation [66]. GelMA also maintains unmodified gelatin's temperature-sensitive characteristics when not photopolymerized. Each GelMA concentration has a certain temperature of gelation; GelMA solution starts to gel below this temperature, creating a physically connected hydrogel. By comparison, if the temperature is above the temperature of gelation, this hydrogel becomes a solution [67]. In addition, modification gelatin with glycidyl methacryloyl reported that having higher storage modulus and quicker enzyme degradation than a standard modified GelMA macromer hydrogel [63].

The strategy used in this study is to evaluate the effect of modification of gelatin with different precursors, in order to elucidate the differences between (MA) and (GMA) functional groups in gelatin and also to determine an optimum

concentration, pressure, temperature of printability assay and hydrogel physical properties including degradation and swelling. This thesis is discussed in the following manner: First, the experimental materials and methods are introduced in detail. Second, the chemical structures of GelMA and GelGMA were characterized by ^{1}H nuclear magnetic resonance (^{1}H NMR). Third, the effect of physicochemical properties are investigated, including swelling, degradation, and printability assessment.