GELATIN METHACRYLAMIDE HYDROGELS FOR THREE DIMENSIONAL BIOPRINTING OF SKIN

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DECLARATION

I, **Ms. Sanjukta**, hereby certify that I had personally carried out the work depicted in the dissertation entitled, **"Gelatin Methacrylamide Hydrogels for Three Dimensional Bioprinting of Skin",** under the direct supervision of Dr. Shiny Velayudhan, Scientist D, Division of Dental Products, Department of Biomaterials Science & Technology, Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences & Technology, Thiruvananthapuram, Kerala, India except where due acknowledgment has been made in the text. No part of the thesis has been submitted for the award of any other degree or diploma prior to this date.

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To whomsoever it may concern

This is to certify that **Ms. Sanjukta** has fulfilled the requirements prescribed for the MPhil degree of the Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram. The dissertation entitled, **"Gelatin Methacrylamide Hydrogels for Three Dimensional Bioprinting of Skin"** was carried out under my direct supervision. No part of the thesis was submitted for the award of any degree or diploma prior to this date.

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Dr. Shiny Velayudhan

The dissertation entitled

GELATIN METHACRYLAMIDE HYDROGELS FOR THREE DIMENSIONAL BIOPRINTING OF SKIN

Submitted by

Ms. Sanjukta

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Dedicated to

MY TEACHERS AND MY FAMILY

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ABBREVIATIONS

3DBP	Three-Dimensional Bioprinting
AGE	Allyl Glycidyl Ether
CAD	Computed Aided Design
CB	Carbonate-Bicarbonate
DLP	Digital Light Processing
ECM	Extra cellular matrix
FBS	Fetal Bovine Serum
FDA	Fluorescein Diacetate
FTIR	Fourier-Transform infrared spectroscopy
GelAGE	Gelatin-Allyl Glycidyl Ether
GelMA	Gelatin Methacrylamide
GelNB	Gelatin-Norbornene
GelSH	Thiolated Gelatin
GelT	Gelatin- Tetrazine
HNMR	Proton Nuclear Magnetic Resonance
LAB	Laser Assisted Bioprinting
MAA	Methacrylic anhydride
MEM	Minimum Essential Medium
PBS	Phosphate Buffered Saline
PCL	Polycaprolactone
PEG	Polyethylene Glycol
PEGDA	Polyethylene Glycol Dimethacrylate
PI	Propidium Iodide
RGD	Arginine-Glycine-Aspartate
SLA	Stereolithography
TNBS	Trinitrobenzenesulfonic acid

CHAPTER 1

INTRODUCTION

Skin is the largest organ of the body and the outermost protecting layer of human body which is always in direct contact to the external environment and thus is more prone to injuries or diseases. As a result of this immediate and promising therapeutic intervention is required when it comes to skin injuries. Three dimensional bioprinting (3DBP) is a promising technique in the field of tissue engineering (TE) which has greatly advanced in last few years. Three dimensional bioprinting can generate controlled organised constructs that resemble the complex native tissues. The printing entity in 3DBP known as the bioink consists cells and biomaterials that produce these tissues. The most commonly used bioink are hydrogels of natural polymers as well as of synthetic polymers. These hydrogels can be used as an efficient bioink for 3DBP. But for skin printing the material to be used as bioink must be able to exist in two different phases as skin is a multi-layered organ with two prominent layers: the epidermis and the dermis. Both the epidermis and dermis are composed for different cells. Thus an efficient bioink for skin printing must be one which can form both the layers of the skin.

Gelatin based hydrogels have excellent properties such as they are highly biocompatible and also have great biodegradability and thus are highly used as bioinks. In addition to these, modification of gelatin can help in tuning the physical and chemical properties of gelatin.One such modification of gelatin with methacrylic anhydride leads to the formation of a modified gelatin- Gelatin Methacrylamide (GelMA). Gelatin methacrylamide has gained severe attention in the field of biomedical technology as a bioink for 3DBP due to its excellent mechanical and biological properties. In this present

study GelMA hydrogel of two concentrations is used to evaluate the physical and biological properties of GelMA as an efficient bioink for skin printing.

Chapter two of the thesis gives reviews of the literature and gives an overview on bioprinting technologies, various bioink systems and their properties. The chapter also touches upon gelatin based hydrogels in bioprinting and several modifications of gelatin. The gap area, hypothesis and the objectives of the current study is also given in this chapter

Chapter three describes the materials and methods used for the study. It details the synthesis of and characterization of GelMA, evaluation of biological properties of the hydrogel and touches on the printability parameters of the hydrogel.

Chapter four gives the results obtained from this study and the discussion of the results. The synthesis of GelMA, its characterization and also the results of the biological properties studied. The most efficient way of synthesising GelMA is by dissolving gelatin in carbonate-bicarbonate (CB) buffer rather than in phosphate buffered saline (PBS). This results in GelMA with higher yield and higher degree of modification. The characterization of GelMA with FTIR and NMR results in the characteristic peak which indicates the interaction of gelatin with methacrylic anhydride and helps in quantifying the degree of modification of gelatin respectively. The TNBS assay also helps in determining the degree of modification of gelatin. The results of various other studies conducted using the two concentrations of GelMA also reveals that both the concentrations of GelMA has good mechanical properties, swelling characteristics, porosity and also are non-toxic to cells as well as can be casted as dual layer constructs.

Chapter five gives a summary and conclusion of the entire study conducted on GelMA based hydrogels for bioprinting of skin constructs. It also provides possible future perspectives on this bio-fabricated skin model.

CHAPTER 2

LITERATURE SURVEY

2.1. BIOPRINTING

Bioprinting is a process involving layer by layer deposition of cells-laden biomaterials in a predetermined structural architecture to generate functional organs. Three dimensional bioprinting (3DBP) is one of the latest technologies which has made it possible to regenerate damaged organs or tissues into functional organ or tissue. This technique involves biomaterials, cells and controlled motor systems for the fabrication of complex structures such as tissue engineering scaffolds, biomedical devices and tissue models. Such models are developed in computer-aided-design (CAD). The advantages of bioprinting include accurate control of cell distribution, high-resolution cell deposition, scalability, and cost-effectiveness. For these reasons the development and subsequent applications of bioprinting have greatly increased. The process of bioprinting involves mainly three steps: preprocessing, printing, and post processing. In the first step, preprocessing uses a CAD technique to develop and design a blueprint of tissue or organ. In the second step, the blue printed design is printed using a bioprinter. Final process involves the tissue maturation and tissue regeneration of bioprinted construct in a bioreactor (Hospodiuk *et al*. 2017).

There are different types of bioprinting such as inkjet bioprinting, laser- assisted bioprinting (LAB), extrusion bioprinting and stereolithography bioprinting. Inkjet bioprinting is a noncontact printing process that deposits precise pico liter droplets of bioink onto a hydrogel substrate *(*Saunders and Derby, 2014*)* Figure 2.1. The common methods are again classified into thermal and piezoelectric actuator methods based on the droplet actuation mechanism.

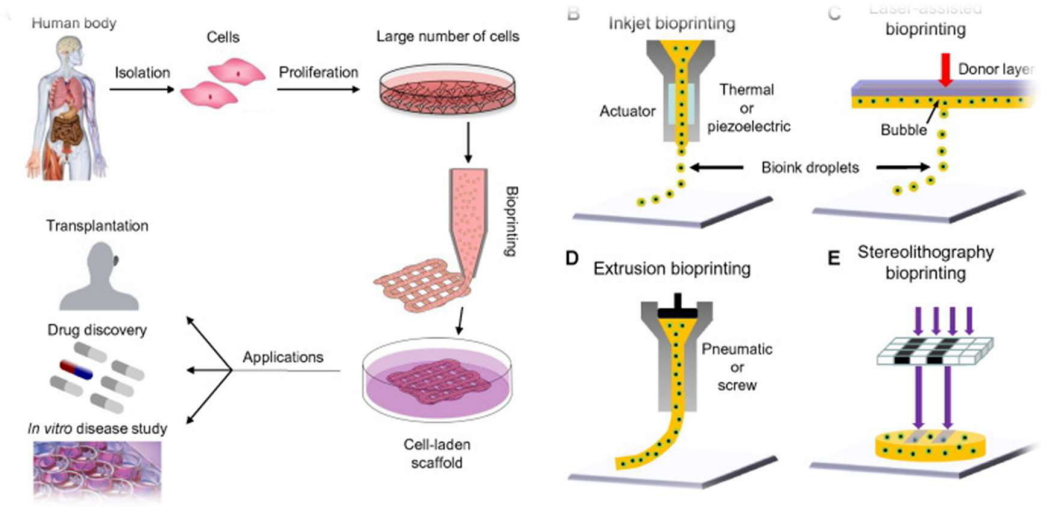

Figure 2.1 Schematic Representation of 3D Bioprinting

Laser-assisted bioprinting uses a laser as the energy source to deposit biomaterials onto a substrate. This technique usually consists of three parts: a pulsed laser source, a ribbon coated with liquid biological materials that are deposited on the metal film, and a receiving substrate (Guillotin *et al*. 2010). The lasers irradiate the ribbon, causing the liquid biological materials to evaporate and reach the receiving substrate in droplet form. The receiving substrate contains a biopolymer or cell culture medium to maintain cellular adhesion and sustained growth after transfer of cells from the ribbon. Laser-assisted bioprinting mainly uses nanosecond lasers with UV or near-UV wavelengths as energy sources to print hydrogels, cells, proteins and ceramic materials. Extrusion printing is a modification of inkjet printing. Inkjet printing cannot print viscous materials thus extrusion printing is developed which uses either an air-force pump or a mechanical screw plunger to dispense bioinks (Pati *et al*. 2015). By applying a continuous force, extrusion printing can print uninterrupted cylindrical lines rather than a single bioink droplet. Stereolithography – more commonly referred to as SLA 3D printing – is one of the most popular and widespread techniques in the world of additive manufacturing. It works by using a high-powered laser to harden liquid resin that is contained in a reservoir to create the desired 3D shape (Wang *et al*. 2016).

2.2. BIOINK

The main component of the 3DBP is the bioink, which is crucial for the development of functional organs or tissue structures Figure 2.2. Bioinks are referred to as cell-laden fluid materials that may have additional containing matrix components, and they

are loaded into the 3D printers for fabricating tissue-like constructs (Whitford and Hoying, 2016). The bioinks require certain properties to provide high resolution during printing and also these properties of a bioink are essential to ensure proper functionality of the printed tissues. The properties of a bioink include printability, mechanical properties, and presence of modifiable functional groups on the surfaces. Biological requirements mainly include biocompatibility and biodegradability (degradation into non-toxic molecules). In addition to these the bioink should also have the ability to retain the 3D printed structure after printing. Two important categories of bioink are used in 3DBP. One is the cell-scaffold based approach and the other one is scaffold-free cell based approach. In the first the bioink consists of biomaterial and live cells, which are printed to develop 3D tissue structures. Here the scaffold biomaterial degrades and the encapsulated cells grow and occupy the space to form tissue structures. But in the second method the live cells are directly printed in a manner that resembles the normal tissue construct. Although, in the field of tissue engineering and regenerative medicine, a wide range of biomaterials have been developed (Furth *et al.,* 2007), but the compatibility of many of them are not sufficient with existing bioprinting technologies. Thus, the need for the development of other types of bioinks is also required which show promising results towards the development of 3DBP.

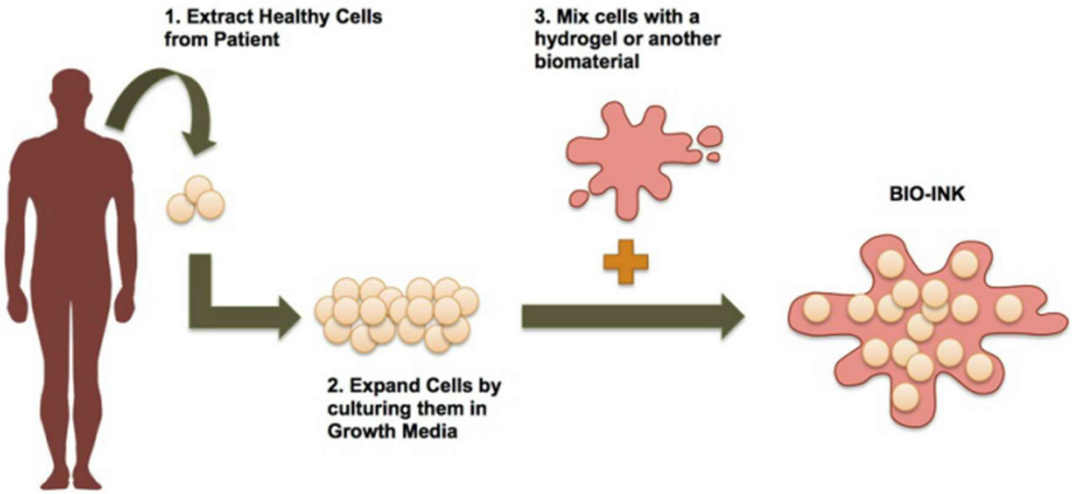

Figure 2.2 Development of a bioink

2.3. HYDROGELS AS BIOINKS

Hydrogels are the crosslinked polymeric substances, which can absorb a large volumes of water compared to their original dry weight (up to 1000 times) without dissolving in the medium (Ahmed, 2015). Hydrogels are the most suitable material to

mimic the native tissue structure using the 3DBP-printing method. Growth factors and nutrients can be delivered with water to the hydrogel network to imitate extracellular matrix environments of the body tissue (Seliktar, 2012).

Based on the origin, hydrogels can be classified into two: naturally derived hydrogels and synthetically derived hydrogels. Hydrogels formed from naturally occurring polymers such as gelatin, fibrin, collagen, chitosan, and alginate are the examples for naturally derived hydrogels whereas hydrogels formed from Pluronic® or polyethylene glycol (PEG) are the examples for synthetically derived hydrogels. Naturally derived hydrogels are the most common hydrogel materials used in tissue engineering applications due to their excellent bioactivity, and their molecular sequences, which are similar to the ECM of natural tissue. However, due to the poor mechanical and rapid biodegradable properties, natural hydrogels are often functionalized with synthetic moieties to improve their stability and robustness.

2.4. HYDROGEL PROPERTIES FOR AN IDEAL BIOINK

To qualify as bioinks, hydrogels should possess ideal biological, physicochemical, rheological and mechanical properties (Figure 2.3). The primary requirement of any hydrogel proposed to be a bioink is its biocompatibility. The hydrogel should also have favorable rheological (printability), mechanical and cell friendly cross-linking chemistry. The degradation profile of the hydrogel should be in sync with the organs being printed.

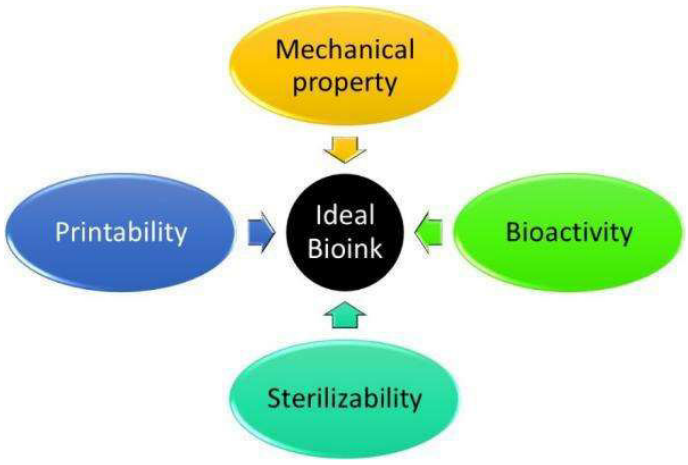

Figure 2.3 Characteristic features of an ideal bioink

2.4.1. Biocompatibility

In the body, cells are present in an extracellular matrix (ECM) consisting of a complex 3D architecture, and they adapt to their surrounding environment by responding

to physical and chemical cues, which have critical implications for cellular function. Materials utilized for 3DBP must be biocompatible and should incorporate biological cues to mediate tissue formation and aid in guiding cell adhesion and proliferation. (Hoffman, 2012). In this context, biomaterials of natural origin are considered ideal candidates as they are based on extracellular matrix (ECM) components such as collagen, hyaluronic acid. However, their intrinsic 'natural' origin and lot-to-lot variability makes these materials very inconsistent and not very reproducible (an essential requirement for reliable research studies). To provide appropriate biomimetic functionality and improve the reproducibility of natural materials in terms of stability and printability many functionalization chemistries are adopted (Section 2.5).

2.4.2. Printability

Printability is an important requirement for the hydrogels to be qualified as bioinks for 3DBP. Printability is defined as the relationship between the bioinks and the substrates which result in an accurate high quality pattern of printing (Paxton *et al.,* 2017). The printability of bioinks can be characterized based on the controllable formation of droplets, jets, filaments or morphology and shape fidelity of the building blocks (**Error! Reference source not found.**).

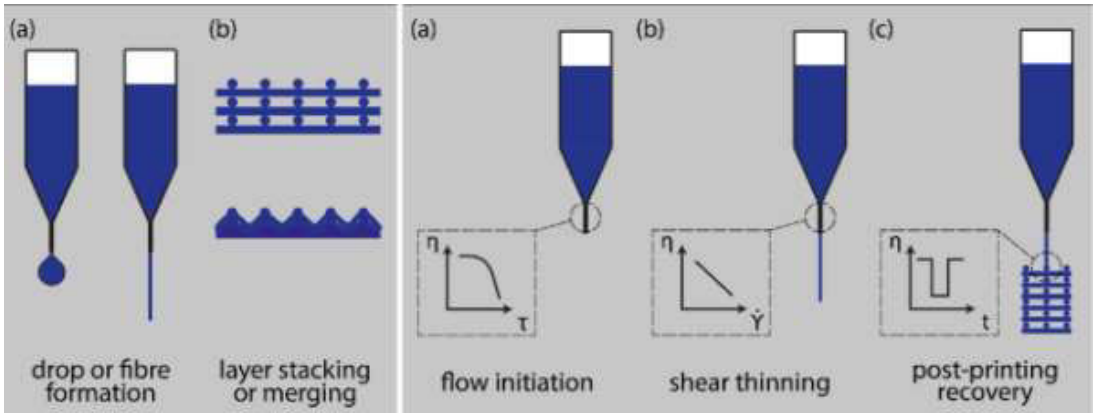

Figure 2.4 Assessing bioink printability (adapted from Paxton *et al* Biofabrication 9 (2017) 044107)

During the initial screening, flow properties of hydrogels are tuned in such a way fiber formation as opposed to droplet formation is established. The fiber forming hydrogels are then successfully stacked in layers without merging between layers. Thereafter, hydrogels are subjected to evaluation for their rheological properties such as (a) the flow

initiation properties and yield stress,(b) degree of shear thinning to predict the extrusion process and cell survival and (c) recovery behavior of the inks after printing (Paxton *et al.,* 2017).

In extrusion-based bioprinting, the hydrogels have a non-Newtonian fluid behavior, that is shear rate and viscosity are dependent on each other (Jungst *et al.,* 2016). The shear thinning and thixotropic behavior of hydrogels also makes it useful for extrusion- based bioprinting because the extrusion of shear thinning hydrogel is easy and its polymer chain become aligned into a favorable direction, by a shear force applied on it. In extrusion based bioprinting, the viscosity can be maintained from 30 mPa/s to 60×10^7 mPa/s (Mandrycky *et al.,* 2016). Depending on the hydrogel concentration, viscosity also varies. For example, Pluronic® F-127 (25%) has a viscosity of 30 mPa/s but when the concentration increases to 40%, the viscosity of Pluronic® F-127 becomes $>600\times10^6$ mPa/s (Chang *et al.,* 2011). Also the attachment of the bioink to the nozzle tip is avoided due to its ideal surface tension properties and low adhesion which enables the formation of filaments and not droplets.

2.4.3. Cross-linking mechanism

Cross-linking of polymer precursors can be undertaken before or after 3DBP to form a hydrogel. All hydrogel polymer chains can cross-link either physically or chemically. Physical cross-linking of hydrogels can be obtained by ionic interaction, hydrogen bonds, protein interaction and crystallization (Yawei *et al.,* 2018, Selcan *et al.,* 2018). Chemical cross-linking is one of the most efficient methods of cross-linking polymer chains to form hydrogels (Valot *et al.,* 2019). In this method a bi-functional cross-linking agent is added to a dilute solution of a hydrophilic polymer which will be having active functional groups to cross-link with the bi-functional agent. Through chemical cross-linking, hydrogels can be prepared from natural as well as synthetic polymers. For example albumin or gelatin based hydrogels are prepared by using dialdehyde as cross-linking agents (Rutz *et al*. 2017). Figure 2.5 lists some of the popularly used cross-linking and reinforcing mechanisms for bioinks.

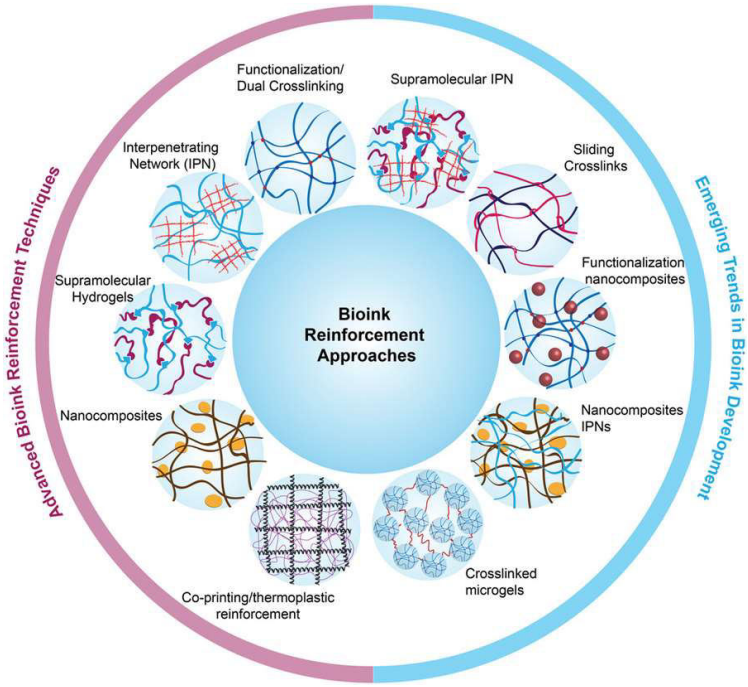

Figure 2.5 Bioink crosslinking and reinforcing modalities, (Valot *et al.,* 2019)

Of the many crosslinking modalities, free radical photopolymerization is one of the commonly used techniques for crosslinking of hydrogels used for bioprinting. Free radical photopolymerization involves the use of photoinitiators that are excited at suitable wavelengths to create free radicals, which in turn promotes the formation of crosslinks between polymer chains.

2.4.4. Biodegradation

Biodegradation means breaking down of a material due to the biological molecules and when a biomaterial is degraded it must degrade into non-toxic products (Nicodemus and Bryant, 2008). Polymers undergo degradation within a body by three means: by oxidation, hydrolytic mechanism and enzymatic mechanism. Gelatin based hydrogels are generally degraded by enzymatic mechanism. The degradation rates of polymers will be thus higher *in vivo* than *in vitro* due to the cellular and enzymatic activities found in body. The type of hydrogels selected, the concentrations, temperature, *in vivo* than *in vitro* conditions and existence of external additives are the factors that depends on the degradability of bioink materials. Thermosensitive hydrogels are extremely sensitive to external circumstances and liquefy easily (Nicodemus and Bryant, 2008). However, the rate of degradation of 3D constructs must be planned according to the uses (Hospodiuk *et al*. 2017).

2.4.5. Sterilizability

Sterilization of any biomaterial or a medical device is important to prevent infections within the body and also to increase the efficiency of the biomaterial or medical device. Despite the beneficial properties and outstanding potential of hydrogels for biomedical applications, several unmet challenges must be overcome, especially regarding to their known sensitivity to conventional sterilization methods. The reports addressing the effects of sterilization on the hydrogels are scarce in literature. One of the commonly adopted methods to ensure sterility of hydrogels is to adopt to aseptic processing/manufacturing techniques. Terminal sterilization is safer and generally involves lower processing costs (Galante *et al.,* 2018).

2.5. GELATIN BASED HYDROGELS AS BIOINKS

Gelatin is a water soluble natural polymer which is obtained from the partial hydrolysis of collagen, a natural polymer found in connective tissues mainly Figure 2.6. Gelatin has helical strands which by self-association lead to form a gel-like material at low *temperatures*. When the temperature increases, it reverts back to a random coil structure (Chiou *et al.,* 2008). Gelatin has less immunogenicity when compared to collagen and promotes cell adhesion, differentiation, migration, and proliferation due to the Arg–Gly–Asp (RGD) groups retained from its precursor *(*Sakai *et al.,* 2009*)*.

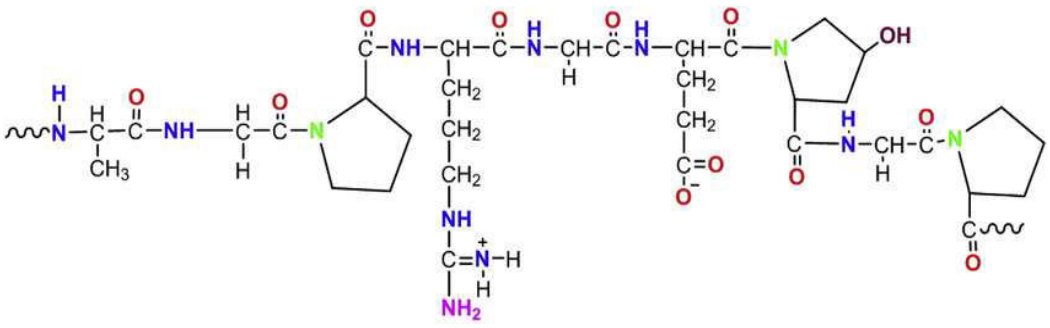

Figure 2.6 Chemical structure of Gelatin

Gelatin based hydrogels have unique features such as excellent biocompatibility, rapid biodegradability and no immunogenicity which makes it useful for organ 3DBP. Gelatin based hydrogels are very popular due to the capability of gelatin solution to be gelled at lower temperature and form hydrogels (Liang *et al.,* 2004). This transition process is known as gelation and during this process the locally ordered regions within the gelatin molecules join together by non-specific bonds. The hydrogels formed as a result of this are

thus thermo-reversible in nature. The unique property of gelatin solution gives uniqueness to gelatin based hydrogels also. Gelatin based hydrogels can be printed and stacked one above the other based on the computer aided designs in a controlled manner. In addition to this gelatin based hydrogels can also be used as wound dressings due to their attractive fluid absorbance property.

2.5.1. Various gelatin modifications for bioinks

The gelatin based hydrogels are extensively used in 3D bioprinting technologies due to their unique physical, chemical, biological and clinical properties. The gelatin based bioinks can be printed in two ways, either as sacrificed bioinks for channel or pore creations (Bertlein *et al.,* 2017) or as solid constructs for cell survival procedures (Wenz *et al.,* 2016). The extrusion based 3D bioprinting technologies have advanced in using gelatin based hydrogels as bioinks for the construction of solid structures that can mediate physical, chemical and physiological functions. But there are certain disadvantages of gelatin. Such as gelatin has a poor thermostability, that is, gelatin becomes a solution at a temperature above 37°C due to cleavage of the hydrogen bonds. Also chemical crosslinking of gelatin can affect the biocompatibility of gelatin as some crosslinking agents can be toxic to the cells. However side chains of gelatin have huge active groups such as –OH, -NH2, -COOH etc. therefore a possible approach is to modify gelatin with certain specific groups to overcome its limitations.

2.5.1.1. Modification with methacrylic anhydride

One of the most common modifications of gelatin results in gelatin methacylamide (GelMA) which is produced by the reaction of gelatin with methacrylic anhydride (MAA).The modified gelatin acquires the ability to photo crosslink due to the presence of methacrylamide groups in it. The solution of this modified gelatin can immediately crosslink in the presence of a photoinitiator under UV light. This crosslinking results in GelMA hydrogels which has excellent thermostability when compared to unmodified gelatin. This modification of gelatin does not compromise with the biocompatibility and degradation property of gelatin and also the GelMA hydrogels supports the cell growth. In addition to this the physical and chemical properties of GelMA hydrogels can be tuned to acquire the gel with characteristics required for various applications. Gelatin methacrylamide hydrogels exhibit excellent properties such as degradability, biocompatibility, and various physical and chemical properties and also is biologically

active. As a result of this GelMA hydrogels are extensively used to study cell responses and can also mimic the native extracellular matrix of cells in cell culture. At a low polymer concentration and a low UV dosage, GelMA hydrogels promoted the microenvironment for mesenchymal stem cells (Pepelanova *et al.,* 2018) Jaipan *et al.,* 2017). Gelatin methacryalmide is a versatile material for wide range of biomedical applications such as tissue engineering (Shin *et al.,* 2016), drug delivery (Lai *et al.,* 2016) and 3D bioprinting applications (Liu *et al.,* 2019).

2.5.1.2. Modification with Norbornene

The modification of gelatin using 5- norbornene-2-carboxylic acid yields to the formation of gelatin-norbornene GelNB (Madl *et al.,* 2020). Gelatin-norbornene hydrogels are very suitable for tissue engineering applications specifically for light-based biofabrication techniques resulting in higher degree of tailorability (Dobos *et al.,* 2019). Gelatin-norbornene can be photopolymerized to form chemically cross-linked hydrogels by means of photoclick chemistry and such step-growth GelNB hydrogels were found to be cytocompatible for in situ cell encapsulation studies (Mũnoz *et al.,* 2014).

2.5.1.3. Modification with Allyl Glycidyl Ether

The reaction of allyly glycidyl ether (AGE) with gelatin under alkaline conditions will result in the synthesis of gelatin- allyl glycidyl ether GelAGE, a thiol-ene photoclickable based hydrogel that can be used as a bioink to meet several demands of biofabrication techniques via lithography - based (digital light processing; DLP) 3D printing and extrusion - based 3D bioprinting, fabricating constructs with high shape fidelity and long term viability of encapsulated cells post printing (Bertlein S *et al*, 2014).

2.5.1.4. Modification with Tetrazine

Gelatin can be modified with 3-(p -benzylamino)-1, 2, 4, 5-tetrazine to get gelatin-tetrazine (GelT). Gelatin- tetrazine can be used as an injectable hydrogels which start to degrade when injected in vivo (Koshy *et al.,* 2016). These hydrogels as bioink can promote high cell viability and have the potential to enhance the formation of elongated morphologies of encapsulated cells (Echave *et al.,* 2019).

2.5.1.5. Modification with Thiol

Gelatin can be chemically modified with thiol groups such as sulfhydryl forming thiolated gelatin (Gel- SH) so that it can be used as an efficient material for biomedical

applications. Free thiol groups can form disulphide bonds within the polymer and these thiol groups are used in strengthening the tertiary and quaternary structure of the protein (Kommareddy *et al.,* 2005). Thiolated gelatin is successfully used for intracellular DNA delivery in response to glutathione and also for the 3D cell entrapment resulting in cell attachment, adhesion and proliferation (Kommareddy *et al.,* 2005, Fu *et al.,* 2012).

2.6. BIOINKS FOR SKIN PRINTING

Bioprinting of skin tissue is essential because such printed skins can be used in drug screening, clinical transplantation, chemical and cosmetic testing as well as basic research. For printing applications, the highly complex skin structure is simplified into two compartments, the epidermis and dermis consisting of two main types of cells, the keratinocytes and the fibroblasts respectively (Yan *et al.,* 2018). A bioink for skin tissue must possess features that are required for cell attachment, spreading, growth and differentiation (Gungor-Ozkerim *et al.,* 2018). Several hydrogel biomaterials such as alginate, gelatin, collagen, fibrin/ fibrinogen, chitosan are developed as bioinks for the 3D printing of skin.

2.6.1. Collagen as a bioink for skin printing

Collagen which is the main structural protein in the extracellular matrix (ECM) of mammalian cells was used as a bioink formulation with encapsulated keratinocytes and fibroblasts for the bioprinting of 3D multilayered skin tissue constructs using laser-based bioprinting technique (Koch *et al.,* 2012). Due to the prevalence of collagen in skin, it is less toxic and has excellent biodegradability which makes it suitable for skin engineering applications. Collagen provides structural and biological support for cells through its fibril structure containing arginine-glycine-aspartic acid (RGD) sequences for cell adhesion. Also collagen-glycosaminoglycan scaffold containing fibroblast and keratinocytes can be used for burn wound treatment.

2.6.2. Fibrin/Fibrinogen as a bioink for skin printing

Fibrinogen is a large, soluble glycoprotein which is involved in blood clot formation where it is converted into insoluble fibrin by thrombin in presence of Ca^{2+} and this fibrin/fibrinogen can be used as a bioink for skin printing due to their biocompatibility and biodegradability in addition to which they induce cell attachment, proliferation and ECM formation (Rajangam and Soo, 2013). Fibrin is also a protein with excellent biocompatibility and biological properties and also the major advantage of using fibrin is

its natural cell binding sites, which do not require further chemical modifications. In recent years, fibrin has been utilized as an injectable biomaterial for the in-situ formation of 3D ECM containing different types of cells such as keratinocytes, fibroblasts and mesenchymal stem cells. Compared to collagen, fibrin provides a mechanically stable extracellular matrix which aids in long-term growth and differentiation of keratinocytes.

2.6.3. Alginate as bioink for skin printing

Alginate is negatively charged polysaccharide that has been used for biomedical applications. The main reasons for the popularity of the alginate are its low cost, simple and fast gelation, and non-immunogenicity, which make it suitable for covering tissues after skin-donating surgery (Yan et al., 2018). As alginate is quickly, but reversibly, crosslinked by $CaCl_2$, it has been employed as a sacrificial template in tissue engineering. The chemically modified alginate can remarkably improve the mechanical stability of the 3D-structure and its double-network composite with synthetic polymers such as polyethylene glycol dimethacrylate (PEGDA) can provide tough and bio-printable 3D-architectures with long term cell survival (Yan *et al.,* 2018).

2.6.4. Chitosan as a bioink for skin printing

Chitosan is a linear polysaccharide with β-(1–4)-linked D-glucosamine and N acetyl-D-glucosamine chemical structure abundantly found in exoskeleton of invertebrates and fungi. Chitosan is a biodegradable, biocompatible, analgesic, and hemostatic polymer, which can be modified as an antimicrobial and anti-inflammatory agent to use as wound healing patches (Yan *et al.,* 2018). Chitosan has been extensively used for cartilage, bone, and skin tissue engineering due to its positive influence on keratinocyte and fibroblast proliferation and adhesion (Yan *et al.,* 2018). However, similar to other hydrogels, it suffers from low mechanical properties and slow gelation; therefore, it should be mixed/copolymerized with the other polymers or crosslinked.

2.6.5. Gelatin as a bioink for skin printing

Similar to collagen, the presence of RGD residues within the gelatin structure promotes cell adhesion, proliferation, and migration. Gelatin exhibits lower antigenicity compared to collagen. The application of gelatin for skin tissue engineering has shown promising results in the promotion of epithelialization and granulation in wound healing. Due to its low mechanical properties, gelatin has been incorporated with other polymers (e.g. polycaprolactone (PCL), poly (lactic acid) and chitosan or crosslinked through

chemical processes or photocrosslinking agents. For example gelatin-alginate composite bioink can be used for the encapsulation of cells for the bioprinting of soft tissue constructs (Zhang *et al.,* 2013). In addition to this, the modified form of gelatin has also been adapted for bioprinting applications such as gelatin methacrylamide. (Nichol *et al.,* 2010).

2.7. GELATIN METHACRYLAMIDE AS BIOINK FOR SKIN PRINTING

In bioprinting, preservation of the integrity and mechanical strength of the bioprinted constructs are two of the most important criteria and gelatin methacrylamide (GelMA) is suitable for meeting these requirements. Cell- laden GelMA bioinks can be bioprinted by using extrusion- based bioprinting technique which results in cell viability and spreading of the printed constructs (Gungor-Ozkerim *et al*., 2018). Gelatin methacrylamide which is a cell-encapsulating hydrogel, creates living tissue structures with precise control over cell attachment and spatial distribution of the cells and also is highly biocompatible with features such as high porosity and ability to retain high water content, allowing the encapsulated cells to receive nutrients and remove waste (Ying *et al*., 2018). Gelatin methacrylamide also allows for network remodeling post printing so that the cells can spread, migrate, proliferate and interact. Gelatin methacrylamide exhibits great biocompatibility for cells due to the presence of RGD peptides. Thus it can be used as an effective bioink for the formation of complex 3D structures with great structural integrity, good shape fidelity and enhanced mechanical stability (Zhuang *et al*., 2019). Skin models fabricated using GelMA as a bioink develop a microenvironment which is closer to the microenvironment of natural skin resulting in a more reliable platform for drug screening, cosmetic testing and various other basic research (Kim *et al*., 2019). Recently an epidermis was fabricated using immortalized human keratinocytes which were embedded within the GelMA, which was equivalent to the natural epidermis and the results demonstrated improved material stiffness for cell adhesion and the formation of stratified epidermis with a certain barrier function (Yan *et al*., 2018). The GelMA which was synthesized for the study had tunable mechanical and degradation properties which were ideal for skin tissue engineering (Zhao *et al*., 2016). According to the results of the study varying the concentration of GelMA prepolymer solution, the physical and biological properties of the resultant hydrogels could be adequately controlled to meet the requirements for epidermis formation (Zhao *et al*., 2016). Gelatin methacrylamide has also been extensively used in epidermal tissue regeneration along with certain other applications as a biomaterial (Liu *et al*., 2017).

2.8. GAP AREA

Gelatin methacrylamide is a widely studied hydrogel for various tissue engineering applications. It has been cited in previous sections the use of GelMA as an established bioink for bioprinting of tissue/tissue constructs. Despite, this the use of GelMA as a bioink for skin is very sporadic. One of the physical challenges of skin bioprinting is the large area of the construct and its dual component called dermis and epidermis. Both the layers are histologically different with respect to the quantity of extracellular matrix and cell types. It is very challenging to bioprint a construct with bioinks having different physical and biological properties. The development of bioink with this dual characteristic has to be developed to address this limitation. Here we explore the feasibility of using GelMA as bioink for bioprinting the dual layer construct. The different concentration of the same bioink will suit to different properties required for the construct.

2.9. HYPOTHESIS

It is hypothesized that bioink containing GelMA at different concentrations can form dual layer construct suitable for skin construct.

2.10. OBJECTIVES

The objectives of this study are

1. Synthesis of Gelatin Methacrylamide
2. Characterizaiton of Gelatin Methacrylamide for its physico-chemical and biological properties
3. Printability analysis of Gelatin Methacrylamide for dual layer printing
4. Biofabrication of Dual layer construct using firbrobalst and HaCaT-laden Gelatin Methacrylamide and evaluation of the biofabricated skin construct

CHAPTER 3

MATERIALS AND METHODS

3.1. MATERIALS

Gelatin (Porcine skin Type A, 175 Bloom), Methacrylic Anhydride (MAA, 94% pure) and 2, 4, 6-Trinitrobenzenesulfonic Acid and 1-[4-(2-hydroxyethoxy) phenyl]-2-hydroxy-methyl-1-propane-1-one (Iragacure 2959) were purchased from Sigma-Aldrich Chemical Company Inc., USA. Sodium carbonate, sodium hydroxide, di-sodium hydrogen phosphate and potassium di-hydrogen phosphate were obtained from Merck, Germany. Sodium chloride and potassium chloride were purchased from SDFCL- S D Fine- Chem Limited, India. Glycine was purchased from Invitrogen by Thermo Fisher Scientific, USA.

Human keratinocytes and fibroblast cell lines (HaCaT and L929) NCCS, Pune, India, Minimum Essential Medium (MEM, Sigma). 10% Fetal Bovine Serum, Penicillin-Streptomycin antibiotic (Pen-Strp 10,000U/ml) and 0.25% Trypsin was obtained from Gibco Invitrogen- Thermo Scientific, Neutral Red Stain, Fluorescein Diacetate (FDA) and Propidium Iodide (PI) was purchased from Sigma, USA

3.2. SYNTHESIS OF GELATIN METHACRYLAMIDE

About 10g of gelatin was weighed and added to 100 ml carbonate-bi carbonate (CB) buffer taken in a 250 ml conical flask, which has been maintained in a water bath at 65°C. A magnetic pellet was also introduced in the conical flask and the contents of the flask were stirred continuously until the gelatin was completely dissolved. Upon dissolution of gelatin the temperature was lowered to 50^0C and 1ml MAA was added in six installments. An amount of 167µl of MAA was added to the gelatin solution at each installment, stirred vigourosly and the pH was monitored. This process was continued until 1 ml of MAA was added to the gelatin solution after which the reaction was stopped and contents dialysed

using a 12-14kDa dialysis tube for 4 days against deionized water at 40^0C. The dialyzed polymer solution (GelMA) was then froze, lyophilized in a lyophilizer (Christ Alpha 1-4 LD, Germany) and stored at -20°C until further use.

3.3. CHARACTERIZATION OF GELATIN METHACRYLAMIDE

The functionalization of gelatin to GelMA was confirmed from the Fourier-transform infrared (FTIR) spectroscopy, Proton Nuclear Magnetic Resonance Spectroscopy (^{1}HNMR) and Trinitrobenzenesulfonic acid (TNBSA) assay.

3.3.1. Fourier-transform infrared spectroscopy

Gelatin, GelMA and cross-linked GelMA were analyzed by FTIR; (Perkin Elmer Spectrum spectrometer). To accomplish this, the samples were fabricated as disc (Φ 8 mm × 2 mm), freeze-dried under vacuum and then tested at 25°C.

3.3.2. ^{1}H Nuclear Magnetic Resonance Spectroscopy

The functionalization of gelatin with MAA was determined by 1H NMR. Briefly, 10 mg of lyophilized Gelatin, and GelMA were dissolved in 1 ml deuterium oxide (D_2O) at 40 °C until a clear solution is obtained. The NMR spectrum was obtained using a Advance Bruker 400 M spectrometer.

3.3.3. Trinitrobenzenesulfonic acid assay

Trinitrobezene sulfonic acid (TNBSA) assay was carried out to quantify the degree of fictionalization of gelatin to GelMA. Briefly, 16mg of GelMA and 16mg of gelatin was weighed out into two glass bottles with screw cap and were allowed to dissolve in 1ml of sodium bi-carbonate at 60°C with continuous stirring. A standard curve was generated using various concentrations (6%, 8%, 10%, 12%, 14%, 16%, 18%, and 20%) (w/v) of glycine from a stock of glycine solution (5.12 mg/10ml). The samples (gelatin and GelMA) and standard were taken in 1.5ml tubes and the total volume was made up to 1ml using sodium bi-carbonate. This was followed by addition of 125µl of TNBS reagent to all the tubes. All the tubes were incubated at 37°C for 2h until a purple colour was developed. After the incubation, the reaction was stopped by adding 62.5µl of 1N HCl, 100µl of the colored solution from each tube was contained in a 96-well plate and the absorbance was measured at 340nm using a microplate reader (Biotech Synergy 4, USA) to measure the fluorescent intensity of the samples. The GelMA DoF can be calculated as follows:

$$DoF\ (Degree\ of\ Functionalization) = 1 - \left[\frac{IGelMA - IDPBS}{IGelatin - IDPBS}\right]$$

where, I_{GelMA}, I_{DPBS} and $I_{Gelatin}$ are intensity of the solution of GelMA, and Gelatin.

All the concentrations of solution used in this study are expressed in (w/v) % unless stated otherwise.

3.3.4. Physical gelling

About 2 ml of GelMA solutions (G_5I_0 and $G_{7.5}$ I_0) were prepared in a test tube by dissolving appropriate amounts of the polymer in Phosphate Buffer Saline (PBS) at 65°C until a clear solution was obtained. The gelling time was noted by tilting the test tube (approximately 45°) until a straight meniscus was obtained. The temperature at which the solution stops flowing was also noted as the gelling temperature.

3.4. FABRICATION OF GELATIN METHACRYLAMIDE HYDROGEL

The hydrogels were prepared by radical cross-linking of solubilized GelMA in the presence of a water-soluble photoinitiator, Irgacure 2959. Two concentrations of GelMA (5% and 7.5 %) were prepared by dissolution in PBS (pH 7.4) at 65°C with continuous stirring A stock solution of Irgacure 2959 was prepared in PBS and mixed with GelMA solutions such that the final concentration of Irgacure in the polymer solution was (0.5%, and 1%). The sample codes against the concentrations of GelMA and Irgacure are given in Table 3.1. The warm mixture was then poured into a glass moulds, cooled to room temperature and immediately exposed to UV light (20mw/cm^2) or a given period of time. A UV lamp (Alphatek Systems, Inc. Kolkata) with 365 nm was used to cure the samples. The cast was stored at 4 °C for a selected period of time after which, a flexible transparent film was obtained.

Table 3.1 The sample codes hydrogels used

Sample Code	GelMA (wt/v)%	Irgacure, (wt/v)%
G_5I_0	5	0
$G_5I_{0.5}$	5	0.5
G_5I_1	5	1
G_5I_2	5	2
$G_{7.5}I_0$	7.5	0
$G_{7.5}I_{0.5}$	7.5	0.5
$G_{7.5}I_1$	7.5	1
$G_{7.5}I_2$	7.5	2

3.4.1. Chemical cross-linking

The gelling time of the GelMA solutions were studied as follows. A 35 mm dish was taken and 1ml from each of the polymer-Irgacure solutions was placed in it and exposed to UV light from a UV lamp procured from Alphatek Inc, Kolkata, until complete cross-linking was accomplished. The crosslinking time was monitored and optimized for each of the concentrations of GelMA and Irgacure.

3.5. CHARACTERIZATION OF GELATIN METHACRYLAMIDE HYDROGEL

3.5.1. Porosity

Gelatin and G_5I_0 and $G7.5I_0$ hydrogels were cast as cylinders of dimensions (ϕ= 2mm and thickness= 5mm), lyophilized and imaged using a desktop cone beam micro CT (μCT, Scanco Medical AG, Switzerland) at 45kVp and 114μA. The voxel size was set at 6μm and the integration time was 0.3s. Approximately, 200 slices per specimen were recorded. Three dimensional images were reconstructed using planar cross-sectional images and the porosity measured.

3.5.2. Swelling studies

The swelling study of G_5I_1 and $G_{7.5}I_1$ were carried out by preparing hydrogel discs of dimensions (ϕ= 4mm and thickness = 1mm) and immersing in them in 1ml of PBS maintained at 37°C. The experiment was carried out in a six-well plate and the change in dimensions and weights of the hydrogel discs were measured at intervals of 24 h, 3 days, 5 days, 7 days and 14 days. On each respective day, the water was wiped out from the surface of the swollen hydrogel sample and the weight was noted. After 14 days, all the samples

were lyophilized and their dry weight was also measured. The percentage swelling was calculated using the equation:

$$Percentage\ swelling = \frac{Ws - Wd}{Wd} \times 100$$

where, Ws – weight of the swollen gel, Wd – weight of the dry weight.

3.5.3. Mechanical properties

The mechanical properties of hydrogels were measured by unconfined, uniaxial compression tests by using an Instron Bioplus 3345 mechanical tester. Cylindrical hydrogel discs (Φ 10 mm × 12 mm) were prepared using 5%, and 7.5% of Gelatin and G_5I_1 and $G_{7.5}I_1$. The samples were then compressed at a rate of 10 mm/min at 25°C until the samples were strained to 50%. Five replicates were tested for each group.

3.5.4. Printability

A single layer filamentous 'S' shape was designed in computer aided design software (FreeCAD). The design was exported as stereolithographic (*.STL) file format which was later imported into a slicing software CURA to generate G-CODE file. The GCODE is then opened in a RepRap application for movement of 3D printer head.

The printability studies were carried out on G_5I_0 and $G_{7.5}I_0$ solutions with the addition of 3% gelatin using a customized 3D bioprinter (Alfatek Systems Inc. Kolkata, India). A 3ml syringe was fixed with a needle gauge and about 2ml of polymer solution was taken in the syringe. The syringe was fixed in the 3D printer and the solution was dispensed at a constant speed to form an 'S' shape which was designed using a computer aided software. The dimensions of the printed structure was analyzed by Image J software for the print fidelity and accuracy.

3.5.5. Biological properties

3.5.5.1. Cell Culture

The cytotoxicity of G_5I_1 and $G_{7.5}I_1$ hydrogels were assessed using keratinocytes (HaCaT Cells) and fibroblasts (L929 Cells) purchased from NCCS, Pune, India. The cells were maintained in Minimum Essential Medium (MEM) supplemented with 10% Fetal Bovine Serum (FBS), 1% Antibiotic (PenStrep, Gi) and 1% L-Glutamine at 37°C in a CO_2 incubator with >90% relative humidity and 5% CO_2. The cells were cultured in T25 flasks (Eppendorf) until a monolayer was formed. The cell monolayer was trypsinized with 0.25%

trypsin and the cells were collected and centrifuged at 1500 revolution per minute (rpm) for 2 min at 37°C. The pellet was the resuspended in culture medium and used for experiments.

3.5.5.1.1 Hydrogel Preparation for Cell Culture Studies

Hydrogels (G_5I_1 and $G_{7.5}I_1$) for cytotoxicity test was prepared in serum-free medium (SFM). The lyophilized G_5I_1 and $G_{7.5}I_1$ hydrogels were weighed out and aseptically transferred to 8 ml SFM and incubated overnight at 37°C. Hydrogel discs of dimension (ϕ = 4 mm thickness = 1mm) were prepared in sterile Teflon mold and subjected to cytotoxicity testing by direct contact. Samples for cell adhesion analysis were prepared by casting the gel directly inside a four-well plate. For cell encapsulation studies the cells were dispersed in hydrogel formulation, transferred to a four-well plate followed by crosslinking with UV.

3.5.5.2. Cytotoxicity by Direct Contact Assay

The cytotoxicity of (G_5I_1 and $G_{7.5}I_1$) hydrogels was tested using direct contact method using HaCaT and L929 cells. Around $1x10^5$ cells were seeded into a 24-well plate and maintained until subconfluency was formed. The culture media was removed and crosslinked G_5I_1 and $G_{7.5}I_1$ was placed on the cell monolayer. G_5I_1 was placed on HaCaT cells and $G_{7.5}I_1$ was placed on L929 cells. The cells were incubated at 37°C in CO_2 incubator for 24h and cytotoxicity was analysed under an inverted phase contrast microscope (Nikon TS 100, Japan). Cytotoxicity was assessed by observing the cells around the material under an inverted phase contrast microscope. The cytotoxic reactivity was assessed based on lysis of cells, cell detachment, morphology and vacuolization. The viability of cells after the direct contact test was determined by neutral red staining. The neutral red stain was prepared in 1.9% NaCl and phosphate buffered saline (PBS) in 1:1 dilution. Approximately 500µl of neutral red stain was added to each well and incubated for 2 min at 37°C. The cells were then visualized under an inverted phase contrast microscope.

3.5.5.3. Cell Viability of Gelatin Methacylamide Hydrogels

The cell viability on (G_5I_1 and $G_{7.5}I_1$) hydrogels was determined by live- dead staining using Fluorescein Diacetate (FDA) and Propidium Iodide (PI). $1x10^5$ cells of HaCaT and L929 cells were added on (G_5I_1 and $G_{7.5}I_1$) hydrogels prepared on a four –well plate respectively. The plates were then incubated at 37°C in a CO_2 incubator for 24-48 h.

The cell seeded on the (G_5I_1 and $G_{7.5}I_1$) hydrogels were then treated with FDA (10µg/ml)/PI (2µg/ml) and visualized under a fluorescent microscope (Leica DMI6000B, Germany).

3.5.5.4. Cell Encapsulation

Cell encapsulation studies were conducted by encapsulating the cells within the (G_5I_1 and $G_{7.5}I_1$) hydrogel. $1x10^5$ cells of, HaCaT and L929 were added to the GelMA solutions of concentration 5% and 7.5% respectively, resulting in the formation of bioink. Approximately 200µl of each bioink was added to 4-well plates and irradiated with UV. After irradiation the plates were incubated at 37°C in a CO_2 incubator for 24 h. The cell viability of encapsulated cells was determined by FDA/PI staining by visualizing under a fluroscent microscope (Leica DMI6000B, Germany) at two time intervals, 0h and 24 h.

3.5.6. Dual Layer Construct

A dual layer construct was cast in a 96-well plate with the lower layer being 7.5% GelMA with L929 cells and the upper layer being 5% GelMA with HaCaT cells.

Prior to dual layer casting, both the cells types were trypsinized and centrifuged to obtain cell pellet. The cell pellets were re-suspended in respective concentrations of hydrogel, that is, HaCaT cells in 5% GelMA and L929 cells in 7.5% GelMA resulting in bioink of two concentrations. After adding 7.5% GelMA bioink, it was irradiated with UV for 2 min and then 5% GelMA bioink was added and a final irradiation with UV was given for 4 min. After adding sufficient amount of 10% FBS media, the plate was incubated at 37°C in a CO_2 incubator for 24-48h. After 48h, the dual layer construct was removed from the well and fixed with 10% neutral buffered formalin for histological studies.

3.5.7. Histology

For the histological analysis the dual layer constructs were embedded in 2% agarose and fixed in 10% neutral buffered saline. The histological analysis included the processing of the material, embedding, staining and then visualization through a microscope.

3.5.7.1. Processing of the Dual Layer Constructs

Before infiltrating with paraffin wax, the dual layer constructs were subjected to a series of alcohol treatment. First the constructs were placed in 80% isopropyl alcohol for 2h, then in 95% isopropyl alcohol for 2h and again in isopropyl alcohol for 1h. After 1h the constructs were placed in 100% isopropyl alcohol for 1h, taken out and again placed in 100% isopropyl alcohol for 1h and then finally in 100% alcohol for 1h. After this the

constructs were placed in xylene initially for 45 min, then again in xylene for 45 min and for 25 min.

After the alcohol treatment the constructs were infiltrated with paraffin wax. The paraffin wax bath temperature was set at 60°C. The processor (Leica ASP300, Germany) was switched on before the processing of the constructs so that the wax will be completely melted in the paraffin step. The infiltration with paraffin was done by placing the constructs in molten paraffin wax for 1h, taking out and then again placing in paraffin wax for 1h and finally placing the constructs in paraffin wax for 2h. The constructs in the casettes are then transferred to the casette bath in the embedder for embedding.

3.5.7.2. Embedding the Dual Layer Constructs

After completely infiltrating the constructs with paraffin wax, the constructs are embedded in a mould to form a block. The mould is also filled with molten wax. The constructs were placed carefully within the mould and a casette is placed on the top of the mould topped with more wax and then this is placed in a cold plate to solidify. After solidification, the block with its cassette is removed from the mould and used for sectioning.

3.5.7.3. Microtomy

For sectioning of the paraffin blocks, a precision saw microtome (Accustom 100, Denmark) was used. Certain parameters such as blade thickness (disposable blade from Leica), section thickness (5µm), blade speed, cutting length and the number of sections were fixed before operating the equipment. Complete sectioning was done and each section was then fixed on a clean glass slide using cyanoacrylate glue.

3.5.7.4. Staining of the Microtomed Sections

The sections of the construct were stained using an autostainer with Heamotoxylin and Eosin stain. Initially the constructs were placed in an oven for 60min to remove the wax and then cooled for 10min. Then the sections were placed in xylene I for 10min, then in xylene II for 5min. After this step, the sections were placed in 90% alcohol for 3min to 5min, then in 70% alcohol for 3mim to 5min. then the sections were washed in tap water for 1min to 3min. Then Harris haematoxylin stain was added to the sections and kept for 30min. After this, the stain was washed with tap water for 1min to 3min. After this wash, the sections were washed with acid alcohol for 5s to 30 s, then again a tap water wash was given for 1min to 5min, then a wash with Scott's tap water was given for 1min to 5min, again a tap water wash for 1min to 5min was given. Then a wash with 70% alcohol was

given for 30s to 2min. Following this wash, 0.5% eosin was added for 1min which was followed by wash with 100% alcohol for 30s to 2min which was repeated twice. Finally the sections were placed in xylene III for 10min to 20min and again in xylene IV for 10min to 20 min.

3.5.7.5. Mounting and Cover Slipping

The slides were removed from xylene and cleaned with tissue paper. The cover slip was cleaned with a clean piece of cloth. A drop of DPX mountant was placed on the cover slip. The mountant is allowed to spread evenly and the cover slip is slowly placed over the glass slide with no air bubbles.

3.5.7.6. Microscopy

The sections of the dual layer constructs fixed on the glass slide were then viewed using a bright field microscope (Nikon 600. Japan) for visualization.

CHAPTER 4

RESULTS AND DISCUSSION

4.1. PREPARATION OF GELATIN METHACRYLAMIDE

The reaction between gelatin and methacrylic anhydride resulted in the production of (GelMA). The reaction is schematically depicted in Figure 4.1.

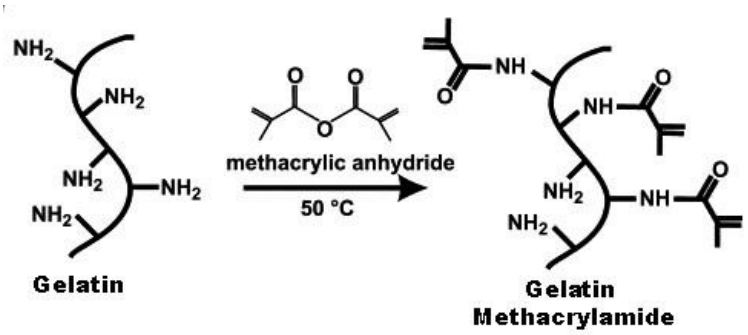

Figure 4.1 Schematics of the reaction of gelatin with methacrylic anhydride

Gelatin is easily functionalized with any anhydride under mild reaction conditions. When MAA reacts with the primary amine groups of gelatin, it dissociates into malic acid and attaches to lysine of gelatin (Van Den Bulcke *et al*., 2000), resulting in addition of methacrylamide groups onto the gelatin macromers (Nichol *et al*., 2010). This method of synthesizing GelMA was originally developed by Van Den Bulcke (Van Den Bulcke *et al*., 2000). They functionalized gelatin to GelMA using MAA at pH 7.4, and reported that their method could carry out ~ 85% functionalization of gelatin. Functionalization reactions of gelatin are pH dependent and lysine based substitution reaction are best conducted at pH >10 (Kenchington, 1958). To achieve a higher degree of substitution, the GelMA synthesis in this study was conducted in CB buffer (pH > 10), as it provides with the required alkaline pH. To maintain the pH > 10, MAA was added at regular intervals and the reaction was

allowed to proceed. A similar study of functionalization of gelatin to gelatin methacryloyl has been reported by Shirahama, *et al.,* (Shirahama, *et al*. 2016), who observed an improved functionalization of gelatin to gelatin methacryolyl upon carrying out the reaction in CB buffer. A schematic illustration of different synthesis processes of GelMA and their respective degrees of substitution is given in Figure 4.2. A similar methodology was adopted in this study, to obtain a better degree of substitution.

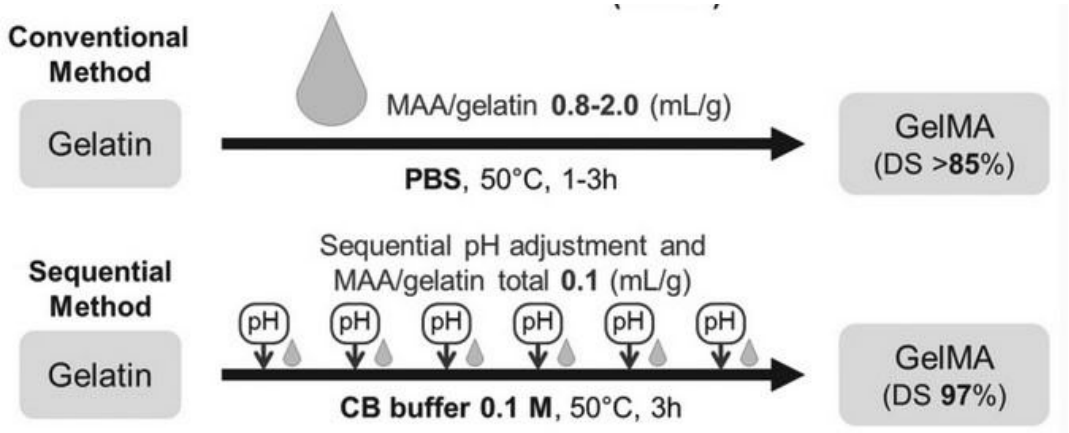

Figure 4.2 Schematic illustration of different synthesis processes of GelMA and their respective degrees of substitution adapted from Shirahama, *et al.* 2016

4.2. CHARACTERIZATION OF GELATIN METHACRYLAMIDE

4.2.1. Fourier Transform Infrared Spectroscopy

The FTIR spectra of GelMA and cross-linked GelMA is given in (Figure 4.3). The spectrum shows the characteristics bands of hydroxyl stretching at 3340 cm^{-1}. The pertinent bands coupled to amide stretching and bending are visible at 1645 cm^{-1} and 1550 cm^{-1}, respectively. The broad band at 3200–3400 cm^{-1} indicates the presence of peptide bonds (mainly N–H stretching). Additionally, the peak at 1680 cm^{-1} indicates the presence of double bonds confirming the successful interaction of gelatin with methacrylic anhydride. The FTIR spectra of cross-linked GelMA is present in Figure 4.3. This spectra shows all the characteristic peaks of GelMA with the exception of the peak at 1680 cm^{-1} that corresponds to a double bond. The disappearance of this peak indicates that GelMA is subjected to UV the cross-linking occurs via the un-saturation.

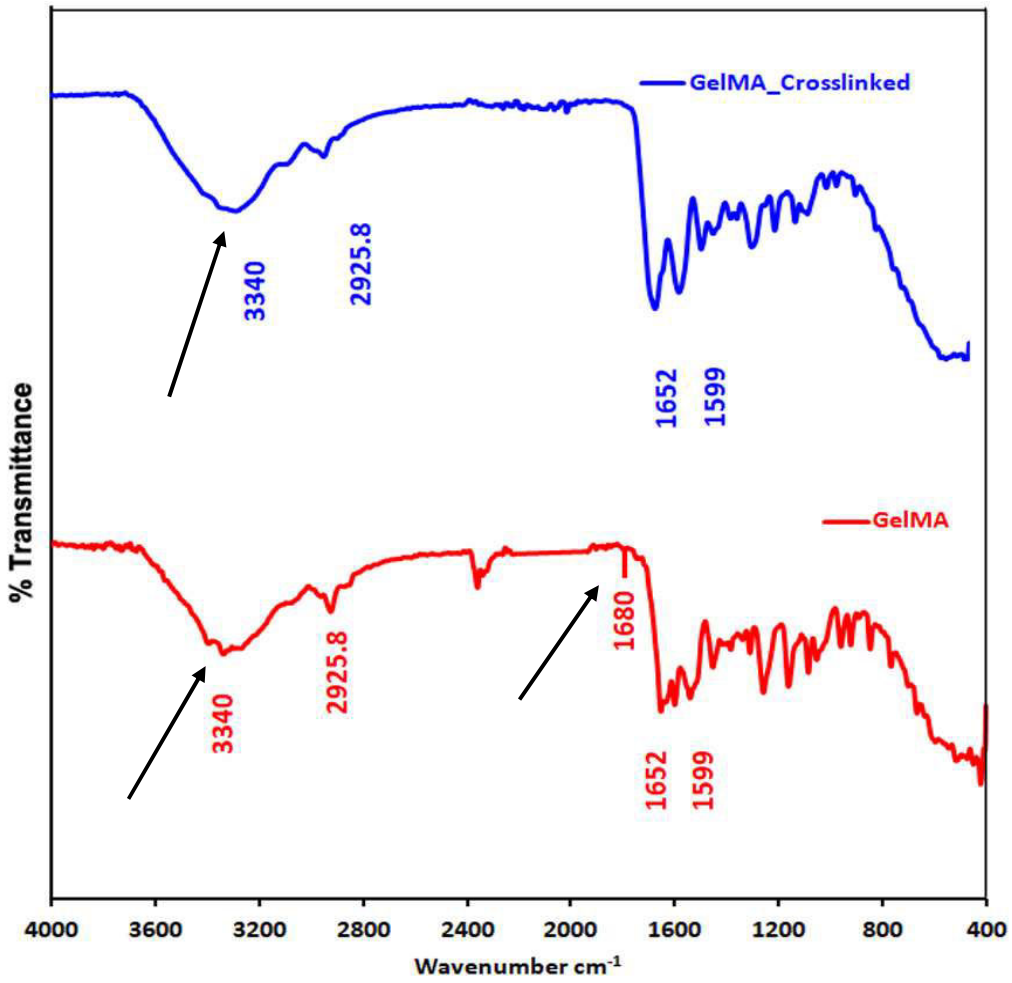

Figure 4.3 FTIR spectrum of GelMA and cross-linked GelMA

4.2.2. ^{1}HNuclear Magnetic Resonance Spectroscopy

Gelatin methacrylamide consists of methacrylamide bonds grafted onto gelatin macromers. The presence of methacrylamide in the gelatin hydrogel was quantified using ^{1}H NMR spectroscopy. The spectrum in Figure 4.4 shows a complex fingerprint from amino acid residues in gelatin and the grafted methacrylamide moieties. The peaks between 5 and 6 ppm on the spectra confirms the presence of acrylic protons from the mecthacrylate, and the peaks between 1.5 and 2 ppm represent the methyl function from the methacrylate (Hoch *et al.,*2012). The lysine methylene from gelatin appears at 2.5 and 3 ppm (Yue *et al.*, 2017). The ^{1}H NMR spectra also confirms the incorporation of the acrylamide double bonds at 5.3 and 5.6 ppm (Yue *et al.,* 2017), which participate in the photopolymerization of GelMA.

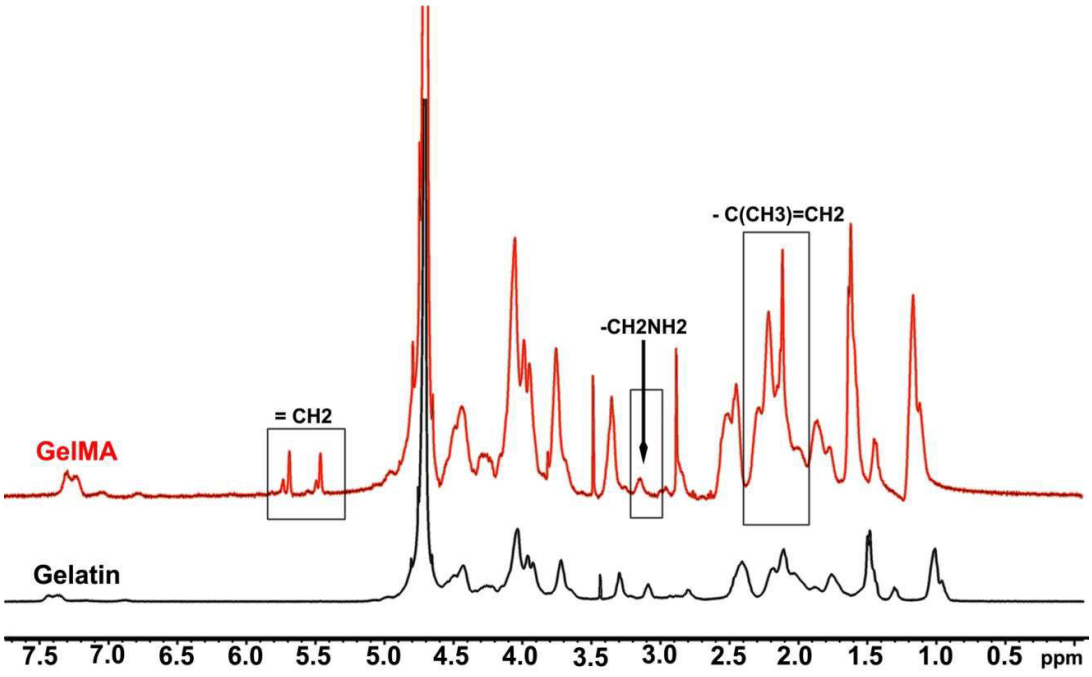

Figure 4.4 ^{1}H NMR spectra of gelatin and GelMA demonstrating successful functionalization of lysine and hydroxylysine residues with methacrylamide groups

4.2.3. Degree of Functionalization

The degree of functionalization of GelMA was quantified by TNBS assay and a standard curve of glycine was used to compare the results. The degree of functionalization was calculated using the standard graph of glycine given in Figure 4.5. The degree of functionalization of GelMA was calculated to be 88.65%.

The degree of functionalization of GelMA depends on the amount of methacrylic anhydride added. The degree of functionalization is important because it affects the pore size, swelling property of the hydrogel and the compressive modulus of the hydrogel (Sun *et al*., 2018). Therefore, synthesis methods which result in higher degree of functionalization are adopted. The synthesis of GelMA by dissolving gelatin in CB buffer result in higher yield and thus higher degree of functionalization (Lee *et al.,* 2015)

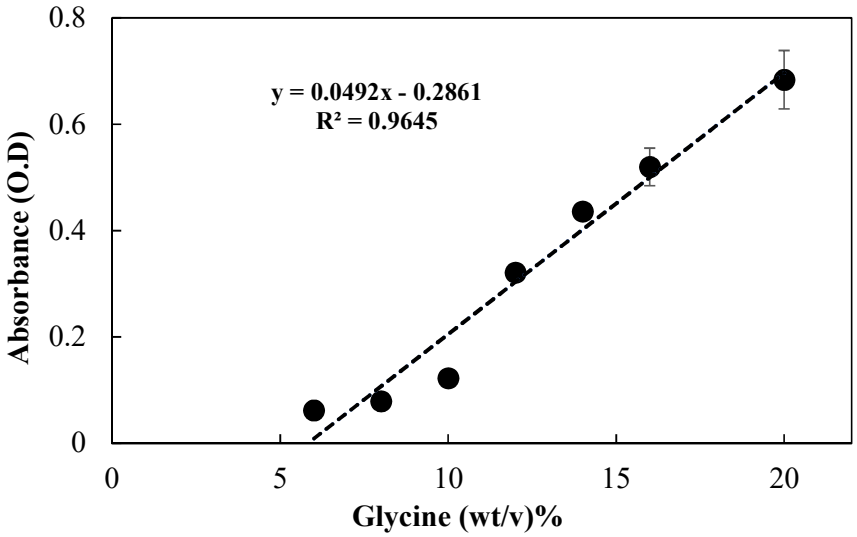

Figure 4.5 Standard Curve of Glycine

4.3. PREPARATION OF GELATIN METHACYLAMIDE HYDROGEL

The water-soluble GelMA can be cross-linked by a number of suitable polymerization processes, such as redox, thermal, and UV treatment; γ-irradiation; or e-beam curing. In this study, cross-linking of is performed by means of UV irradiation (365 nm) in the presence of a water-soluble photoinitiator. Upon absorption of UV light, the photo-initiator generates free radicals and GelMA is subsequently polymerized, as shown in Figure 4.9.

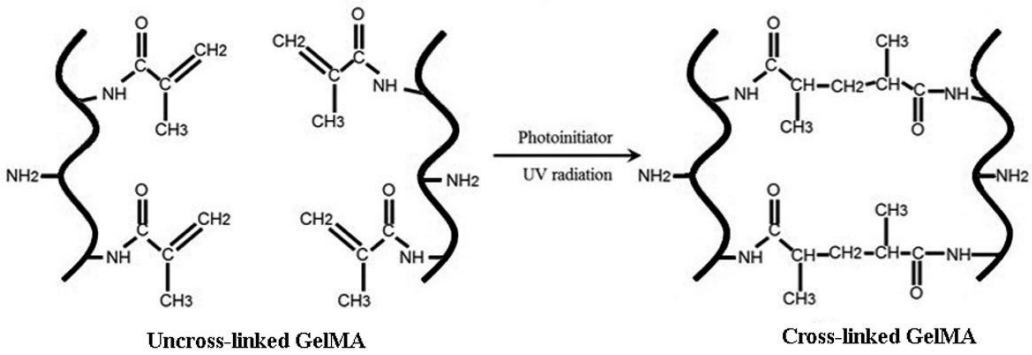

Figure 4.6 Schematic illustration of cross-linking of GelMA under UV irradiation

Typically, not all of the amine groups on gelatin macromers are substituted with methacrylamide bonds.

4.4. CHARACTERIZATION OF GELATIN METHACYLAMIDE HYDROGEL

4.4.1. Physical Gelling of Gelatin Methacrylamide

The physical gelling of gelatin and GelMA solution is described in Figure 4.7 and Figure 4.8. Physical gelling is a temperature dependent process. Gelatin upon cooling, gels at its gelling temperature which is 20°C, but at room temperature, gelatin exists in a liquid state (Rebers *et al.,* 2019). Similarly GelMA does not show any physical gelling at normal physiological temperature but gels at temperatures lower than the gelling temperature of gelatin. This is due to the lower physical interaction in modified gelatin molecules. With increase in degree of modification, the physical interaction reduces. However in solutions with higher concentration of GelMA, physical gelling was observed faster when compared to solutions having lower concentration of GelMA. A group also concluded similar results when they compared the physical gelling of gelatin and GelMA (Van Den Bulcke *et al.,* 2000).

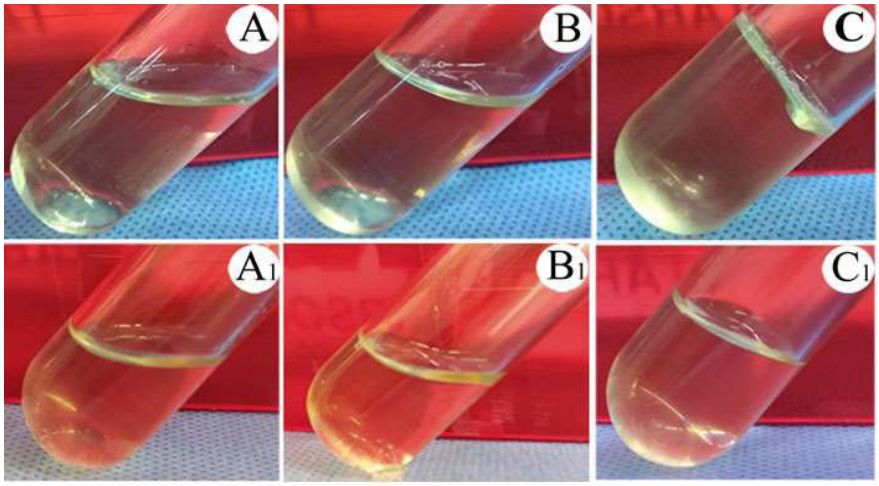

Figure 4.7 Physical Gelling of 5% Gelatin and GelMA solution. At liquid state (A and A1), Onset of Gelling (B and B1), Gelled (C and C1)

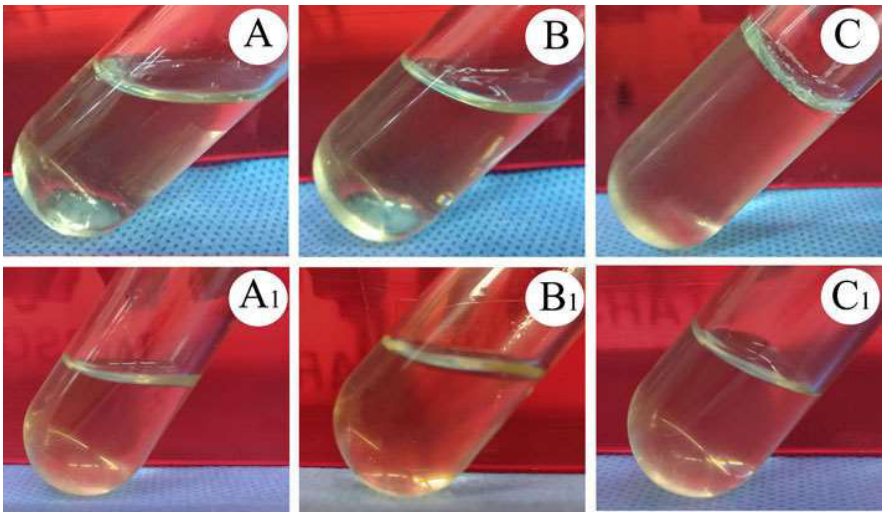

Figure 4.8 Physical Gelling of 7.5% Gelatin and GelMA solution. At liquid state (A and A1), On set of Gelling (B and B1), Gelled (C and C1)

4.4.2. Chemical Crossslinking of Gelatin Methacrylamide

The crosslinking time of GelMA with respect to the concentration of the photoinitiator is given in Figure 4.9. From the figure, it is clear that higher the concentration of Irgacure, lower will be the crosslinking time.

Crosslinking with UV light usually relies on photoinitiators such as Irgacure. Ultraviolet light photons cause dissociation of the photoinitiator into radicals which results in radical formation of methacrylamide groups. Since these radicals are highly unstable they combine with each other and form a crosslink network. Minimum exposure of UV is required as longer exposure of UV can cause damage to the cells. Thus Irgacure concentration is increased to fasten the crosslinking process. Concentration of Irgacure in the range of 0.05% to 1% is reported to be non-cytotoxic to the cells.

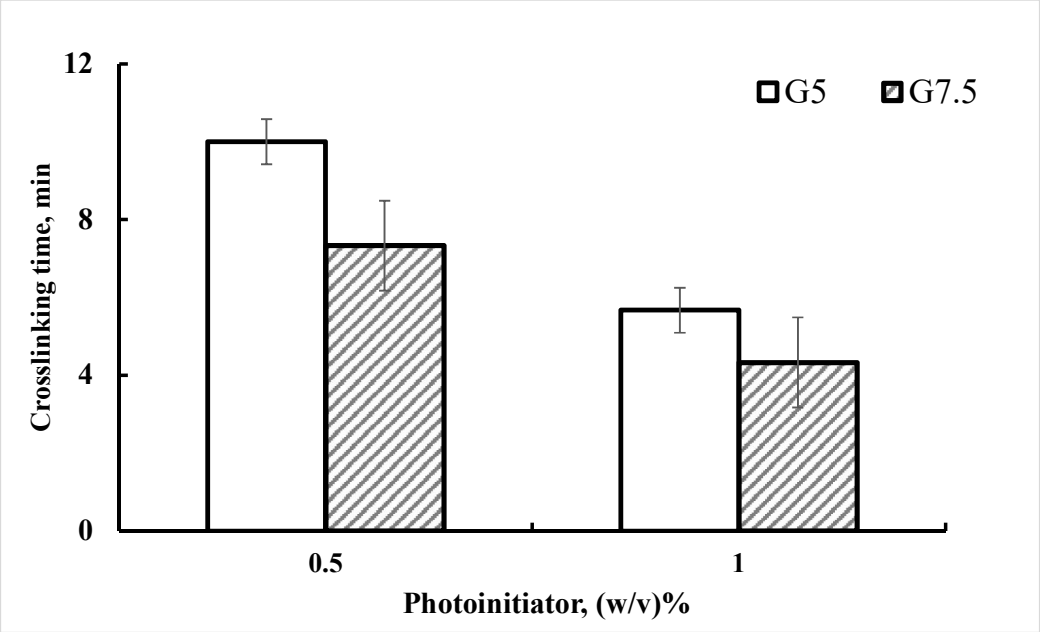

Figure 4.9 Crosslinking time of GelMA with respect to Irgacure concentration

4.4.3. Swelling Characteristics of Gelatin Methacrylamide

Swelling is an indicator of the amount of water sorption into a hydrogel (Zhao *et al*., 2016). In 3D bioprinting applications, cells are typically encapsulated within the hydrogels. The hydrogel swelling behaviors have direct effects on the nutrient retention in a hydrogel and waste egestion from cells out of a hydrogel. In this study two compositions of GelMA hydrogels (G_5I_1 and $G_{7.5}I_1$) were subjected to swelling in PBS was performed on GelMA hydrogels for a period of 14 days. The Figure 4.10 depicts the results of swelling studies carried on hydrogels. The swelling ratio of the G_5I_1 was greater than that of the $G_{7.5}I_1$. This is expected because the swelling ratio of a hydrogel is inversely proportional to the concentration of the polymer. The hydrogel fabricated from $G_{7.5}I_1$ exhibited a total percentage swelling of 60, were still structurally stable and did not disintegrate. While the hydrogels fabricated from G_5I_1 remained stable only for 5days, after which they disintegrated. The swelling property of hydrogels depend on their pore size and the interaction between the polymer and the solvent (Nichol *et al.,* 2010). The swelling ratio decreases with increase in GelMA concentration because higher concentration of GelMA will have higher crosslinking density which would affect the swelling behavior of the hydrogels (Yoon *et al.,* 2016).

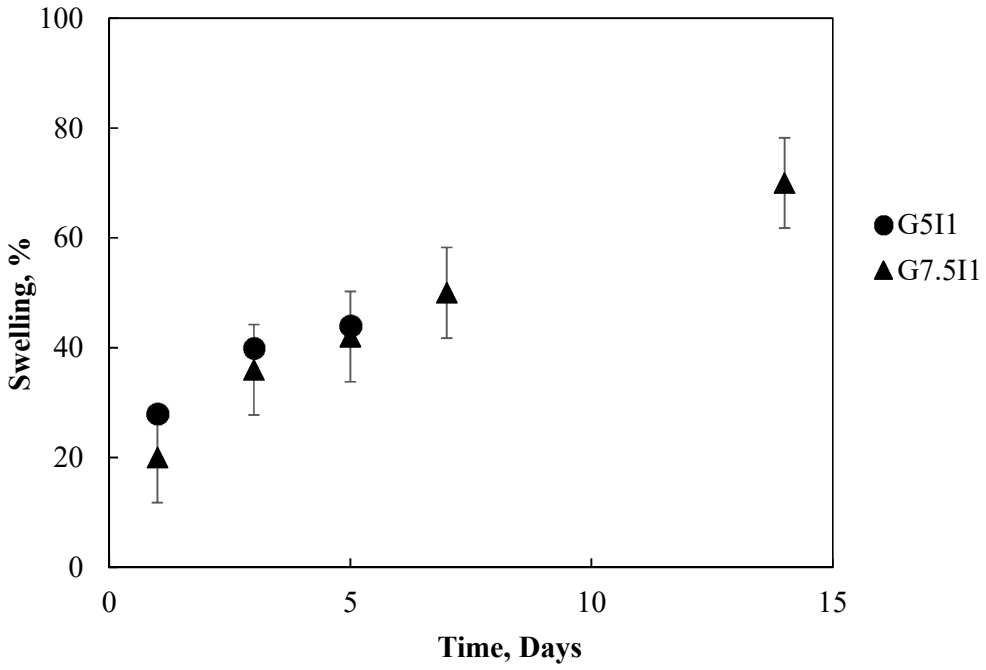

Figure 4.10 Variation of swelling of G_5I_1 and $G_{7.5}I_1$ with time

4.4.4. Porosity

The porosity analysis of the G_5I_1 and $G_{7.5}I_1$ were carried out on lyophilized samples using micro CT. The generated 3D model (Figure 4.11) demonstrated a morphology, pore size, shape, and distribution of G_5I_1 and $G_{7.5}I_1$ lyophilized samples. While both G_5I_1 and $G_{7.5}I_1$ showed almost homogenous morphology in general, $G_{7.5}I_1$ exhibited the presence of larger pores on the surface. These non-homogenous distribution of pores could be a result of the inherent limitation associated with the lyophilization technique.

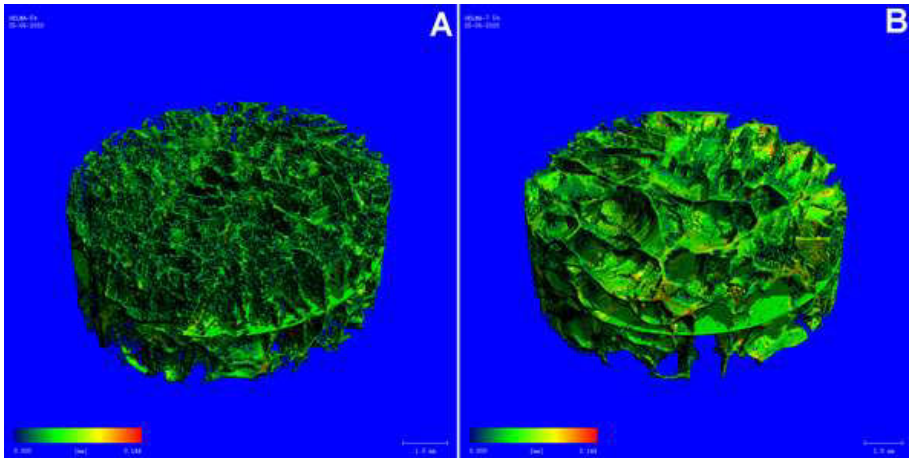

Figure 4.11 3D model of the G_5I_1 and $G_{7.5}I_1$ foam generated through the micro-CT

Micro-CT analyses also enabled investigating the average pore size distribution (Figure); most of the foam pores had a diameter size in the range of 150 - 400 μm which was adequate for any kind of tissue engineering applications (Karageorgiou and Kalpan, 2005).

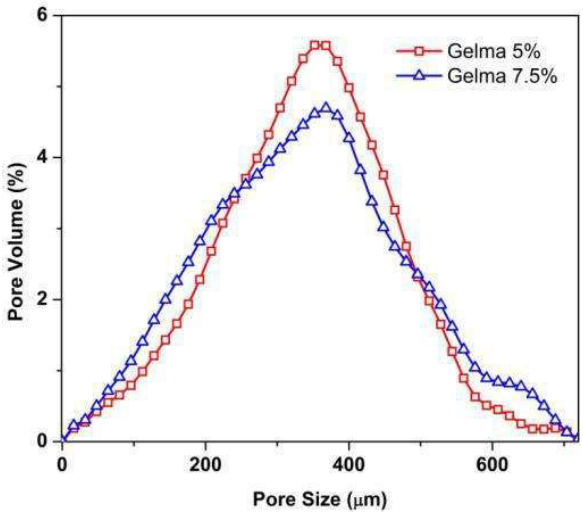

Figure 4.12 The histogram of G_5I_1 and $G_{7.5}I_1$

4.4.5. Mechanical Properties of Gelatin Methacrylamide

Figure 4.13 gives the representative stress-strain curves of gelatin and G_5I_1 and $G_{7.5}I_1$. It can be seen from the figure that G_5I_1 displays a lower mechanical performance when compared to $G_{7.5}I_1$. This is expected because the mechanical properties of hydrogels mainly depend on the original rigidity of polymer chains, molecular weight of the polymer, the crosslinking density and the concentration of the polymer (Lee and Mooney, 2001). The hydrogel G_5I_1 has a lower polymer concentration than $G_{7.5}I_1$. In the case of GelMA hydrogels, degree of methacylation and the UV exposure time also influences the mechanical properties. This study utilized GelMA polymers with a degree of methacrylation of 88.6% and the exposure time to UV were kept constant during fabrication of the hydrogels. Hence the difference in the mechanical properties observed is purely due difference in the polymer concentrations used. The mechanical properties of G_5I_1 and $G_{7.5}I_1$ were however, found inferior when compared to similar concentrations of virgin gelatin. This could be due to the possible degradation of gelatin molecules during the process of methacrylation.

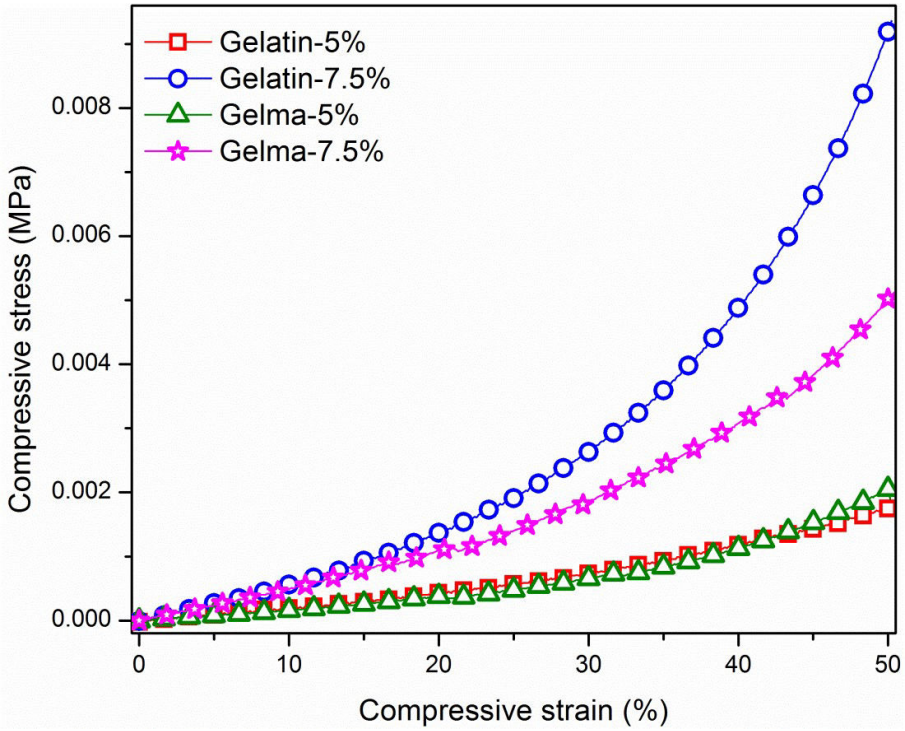

Figure 4.13 Representative stress-strain curves of Gelatin and GelMA and various polymer concentrations

The compressive modulus of the hydrogels were calculated from the compressive stress-strain curves (Figure 4.14). It is clear from the figure that the moduli of G_5I_1 is lower than that of $G_{7.5}I_1$. Hence, $G_{7.5}I_1$ could be a suitable candidate for the stiff network of a hydrogel that could form the potential layer of the dermis for a skin construct. G_5I_1 on the other hand could form a loose and soft layer that could form the epidermis.

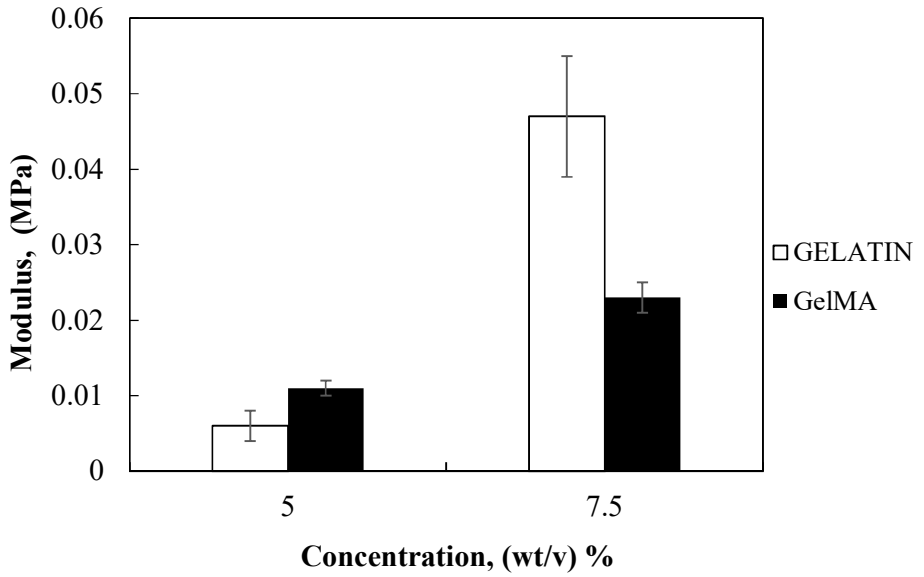

Figure 4.14 Variation of compression modulus of Gelatin and GelMA and various polymer concentration

4.4.6. Printability of Gelatin Methacrylamide Hydrogels

The printability of G_5I_0 and $G_{7.5}I_0$ solutions with 3 % gelatin was assessed by analyzing the printed structure. Based on the GCODES of the 'S' shape, the line width expected in the construct was 2500 µm with four sharp 90° turns within it.

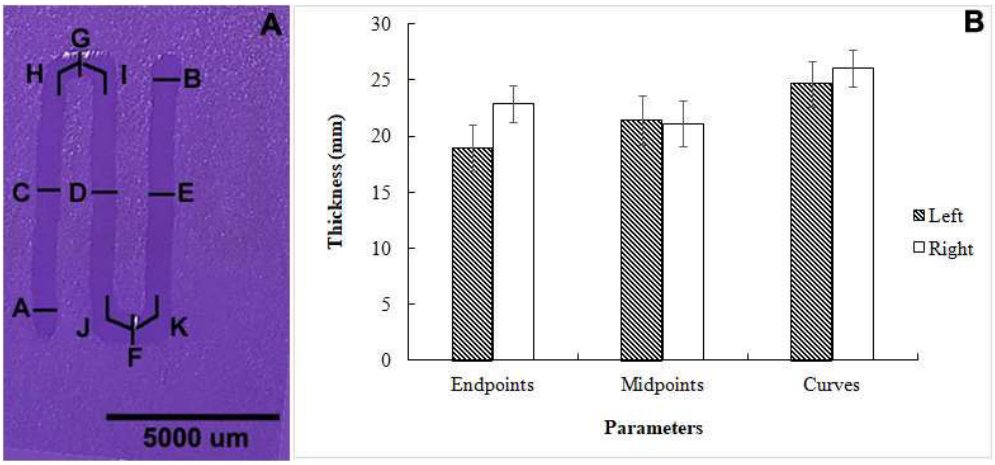

Figure 4.15 Printability of 5% Gelatin methacrylamide hydrogels.

The 'S' shape CAD design was exactly translated by extruding both 5% and 7.5 % GelMA hydrogel solutions. The dimensions of the printed structure was analyzed by Image J showed the print fidelity and accuracy. The S structure was categorized as Endpoints, Midpoints and Curves for the analysis (Figure 4.15 and Figure 4.16). Both 5% and 7.5% GelMA appeared exactly in the same dimensions as designed. The filament width

was between 2000 -2500 µm in the case of 5% GelMA where as it was 2000 to 3000 µm in the case of 7.5% GelMA

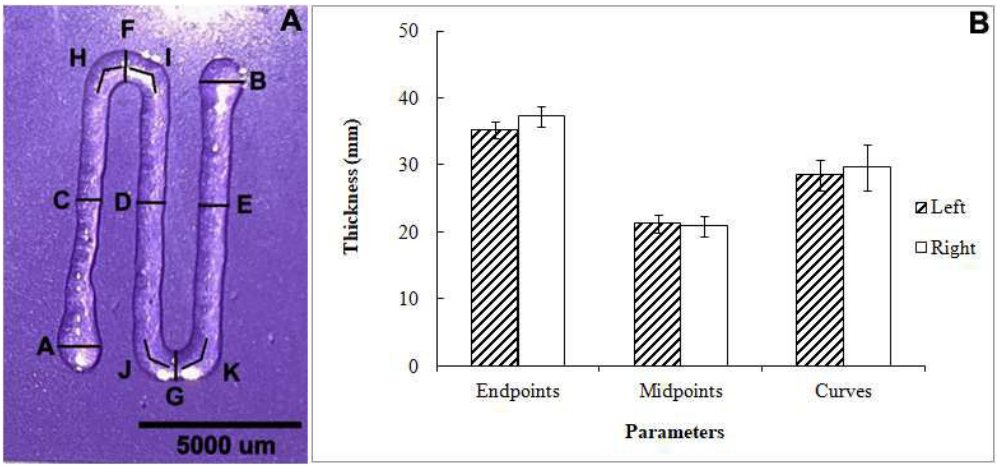

Figure 4.16 Printability of 7.5% Gelatin methacrylamide hydrogels

4.4.7. Biological Evaluation of Gelatin Methacrylamide Hydrogels

4.4.7.1. Cell culture

The HaCaT cells and L929 cells maintained in MEM media with 10% FBS attained sub-confluent state and were used for further experiments. The monolayer of cells formed is shown in the Figure 4.17

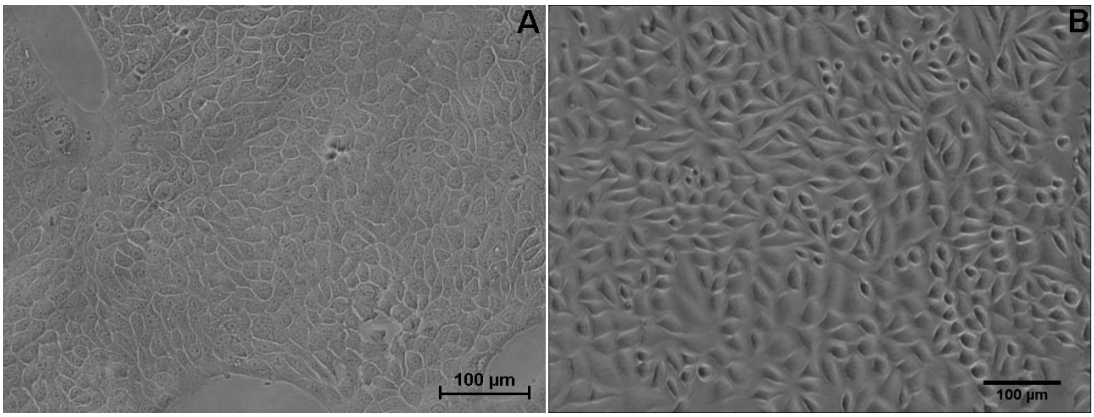

Figure 4.17 Monolayer of (A) HaCaT and (B) L929 cells

Cytotoxicity is a preliminary test required to test the potential toxic response of the material to the biological system. In the direct contact method, potential cytotoxic responses around the G_5I_1 and $G_{7.5}I_1$ hydrogels were analyzed. The cytotoxic reactivity responses such as cell detachment and lysis were absent in both the hydrogels (Figure 4.18) and the cell morphology was similar to the cell control (Figure 4.18).

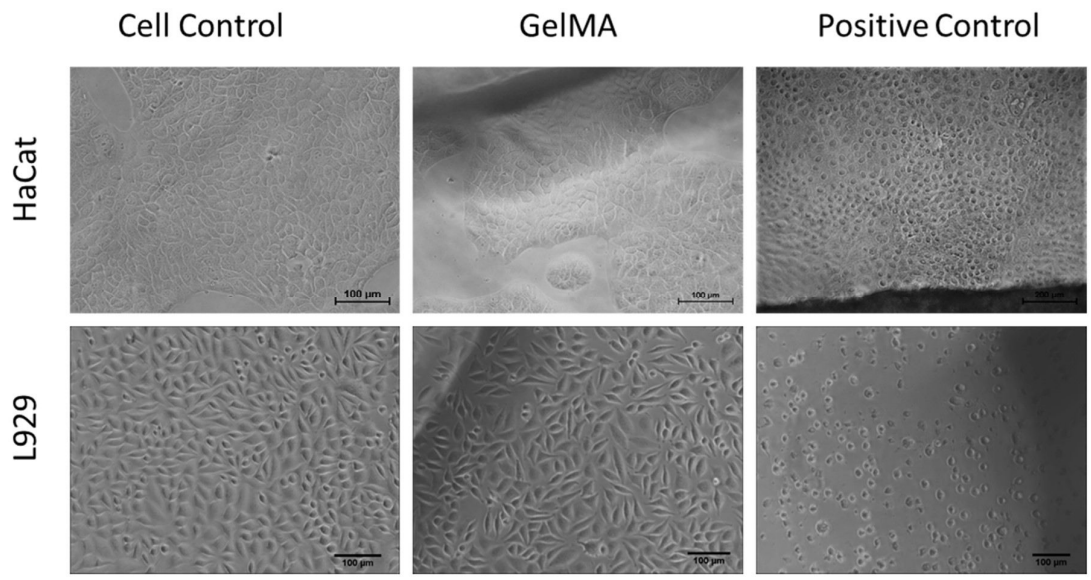

Figure 4.18 Direct Contact Test of G_5I_1 and $G_{7.5}I_1$ hydrogels with HaCaT Cells and L929 Cells

The viability of cells after the direct contact test was confirmed by neutral red staining (Figure 4.19). The positive control showed severe cytotoxic responses (Figure 4.19). The results confirmed that G_5I_1 and $G_{7.5}I_1$ hydrogels were non-toxic to keratinocytes (HaCaT cells) as well as fibroblast cells (L929 cells).The data obtained from the direct contact assay is similar to previously reported data (Drury *et al*., 2003).

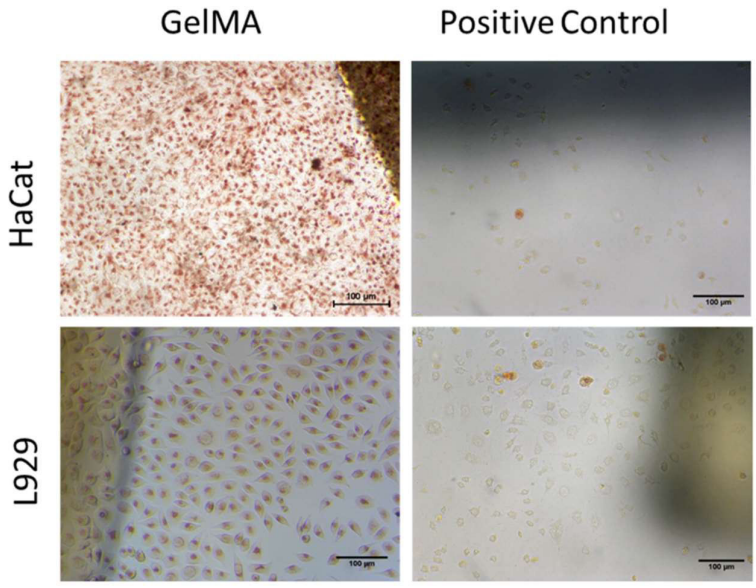

Figure 4.19 Neutral red staining of HaCaT and L929 cells after direct contact with G_5I_1 and $G_{7.5}I_1$ hydrogels for 24 h

4.4.8. Cell Viability of Gelatin Methacrylamide Hydrogels

The interaction of cells to any material is possible when the cell surface receptors interact with the cell binding domain in ECM (Dobkowski*et al*., 1999). The surface topography, wettability, protein adhesion, surface charge and surface roughness significantly influence cell behaviors such as adhesion, spreading and proliferation (Chang and Wang, 2011). HaCaT cells and L929 cells adhered on G_5I_1 and $G_{7.5}I_1$, respectively appeared in round morphology. However, the viability staining by FDA/PI revealed that the cells adhered on respective hydrogels are live. The viable cells appeared green while nucleus of dead cells appeared red (Figure 4.20). Tong *et al* reported that varying hydrogel stiffness affects fibroblast morphology (Tong *et al*., 2016).The composition of the ECM and the substrate stiffness play an important role in cell morphology. Hope *et al* demonstrated that substrate stiffness directly influence the morphology of human dermal fibrobalsts. Cells on softer substrate appeared round while those adhered to stiffer gel showed typical fibroblastic morphology (Hopp *et al*., 2013). Hence it could be deduced from the round morphology and viable cells that, G_5I_1 and $G_{7.5}I_1$ is with soft surface which is in par with the compressive modulus obtained (Figure 4.20).

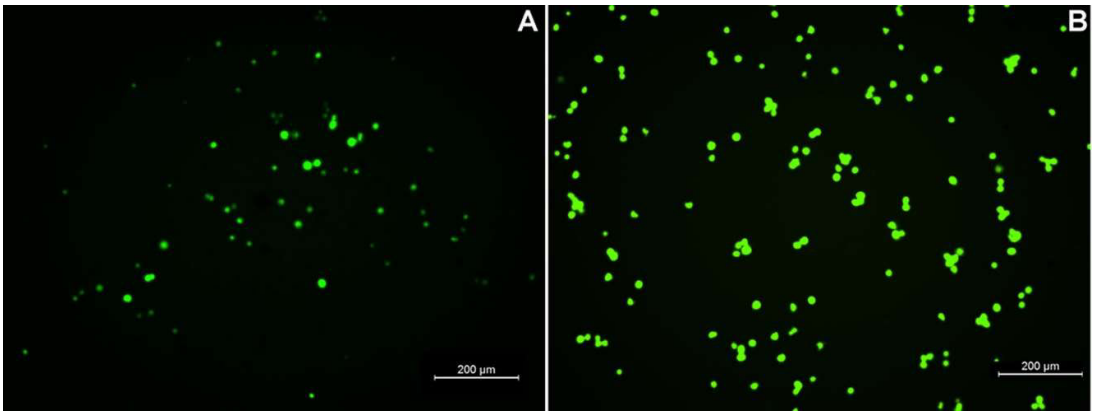

Figure 4.20 Cell viability of HaCaT Cells (A) and L929 Cells (B) on GelMA Hydrogels

4.4.9. Cell Encapsulation/Preparation of Bioinks

The bioinks were prepared by encapsulating HaCaT and L-929 cells in G_5I_1 and $G_{7.5}I_1$, respectively. The viability of encapsulated cells analyzed 24h after crosslinking, by FDA/PI staining showed that cells are viable (Figure 4.21 A, B). Cell viability of both HaCaT cells and L929 cells were more than 90% within the G_5I_1 and $G_{7.5}I_1$ hydrogels. The high cell viability indicated that the presence of photoinitiator as well as the crosslinking process during UV exposure had minimal effect on the cells. Similar results were reported in previously published works (Nicodemus *et al*., 2008).

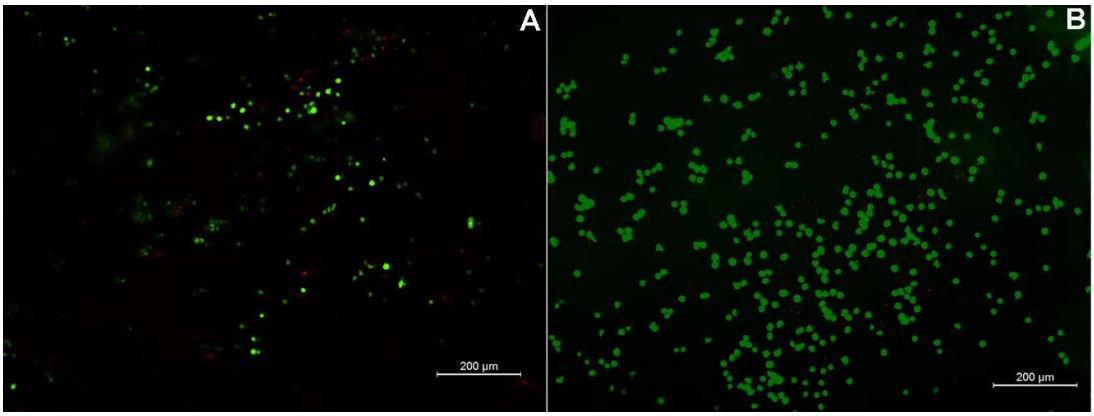

Figure 4.21 Cell Encapsulation of HaCaT Cells (A) and L929 Cells (B)

4.4.10. Histology of the dual layer construct

The results of the histological studies are presented in the Figure 4.22. The figures represent the dual layers of GelMA hydrogels with cells entrapped within them. This shows that GelMA hydrogels (G_5I_1 and $G_{7.5}I_1$) can be casted as two layers with $G_{7.5}I_1$ being the lower layer with fibroblast cells G_5I_1 and being the upper layer with keratinocytes similar to the epidermis and dermis layers of the skin.

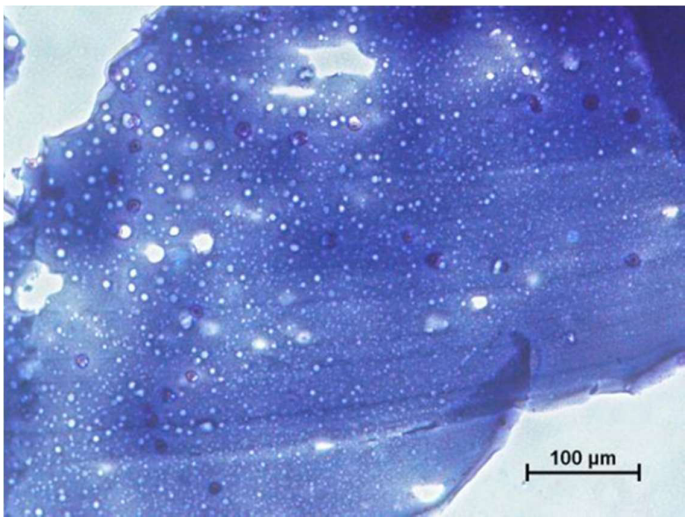

Figure 4.22 Histology of the cells (L929) encapsulation in $G_{7.5}I_1$

CHAPTER 5

SUMMARY, CONCLUSIONS AND FUTURE OUTLOOK

This thesis explores the possibility of using Gelatin methacryalamide (GelMA) as a bioink for printing skin construct. Gelatin methacrylamide is a widely studied and established hydrogel for bioprinting applications and is formed by the reaction of gelatin with methacrylic anhydride. Gelatin Methacrylamide was synthesized and is crosslinked by irradiating with UV in the presence of a photoinitiator Irgacure to form the hydrogel. The reaction is carried out in room temperature and the hydrogel formation was observed within 3 min to 5 min of UV exposure. Two different concentration of the hydrogel was used (G_5I_1 and $G_{7.5}I_1$). Gelatin, GelMA and crosslinked GelMA was characterized by FTIR spectroscopy, swelling studies, mechanical properties, and printability analysis. In addition to this, $G_5I_1$and $G_{7.5}I_1$ were also analyzed by in vitro biological evaluation to know the response of cells towards the materials. The results obtained from the swelling study gave an idea about the water uptake capacity of the hydrogel and that higher concentration of GelMA has lower swelling ratio due to higher crosslinking density. Similarly the mechanical property studied by the compression test revealed that higher GelMA concentration resulted in higher mechanical stability. Printing of $G_{7.5}$ was easier and better than that of G_5 due to the higher viscosity of $G_{7.5}$. The biological evaluation proved that both G_5I_1 and $G_{7.5}I_1$ hydrogels are non-cytotoxic to fibroblasts and keratinocytes when placed in direct contact with cells. The keratinocytes and fibroblasts were viable when seeded on G_5I_1 and $G_{7.5}I_1$ respectively. Cells were also viable when they were entrapped within the hydrogels. The cell suspension prepared in GelMA (Bioink) was used to create a dual layer construct and the histological analysis showed the distribution of cells in the

dual layer. From all the results obtained, it clear that the GelMA hydrogels possess good mechanical as well as biological properties to be used as an efficient bioink for 3DBP.

Skin bioprinting requires cells, biomaterials and other active ingredients to create viable constructs in a controlled manner that can be successfully developed into a multi-layered skin substitute for various advantages in the biomedical field such as for drug testing, cosmetic testing, and clinical transplantation etc. Gelatin methacrylamide can be used as a potential bioink for skin bioprinting. The results of this study reveal certain specific properties that GelMA possess, which can be efficiently utilized in the bioprinting of skin. Another advantage of GelMA is it's suitability for dual layer printing using different concentrations similar to the epidermis and dermis layers of the skin. Thus using GelMA for skin printing can be a promising approach to attain structurally and functionally efficient skin constructs.

Three dimensional bioprinting has great opportunities to build complex skin constructs that can be used to overcome the complicated skin defects which are difficult to heal by normal clinical procedures. GelMA has shown to be a promising bioink for skin bioprinting. The bioprinted dual layer construct will be the future prospects that this study But further studies on GelMA as a bioink and printing of GelMA with growth factors and other active ingredients in addition to the cells is required to create a skin construct which has to be structurally and functionally similar to the native skin with proper vascularization, presence of hair follicles and other appendages. If this study can be completed with positive results then further studies and clinical trials may lead to the development of GelMA as a bioink for the three dimensional printing of skin.

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