Design And Development of Crosslinker Module for Three-Dimensional Bioprinting of Hydrogels

This report is submitted in accordance with requirement of the Universiti Teknikal Malaysia Melaka (UTeM) for Bachelor Degree of Manufacturing Engineering (Hons.)

by

MUAMMAR GHADDAFI BIN MOHD ALI

FAKULTY OF INDUSTRIAL AND MANUFACTURING
TECHNOLOGY AND ENGINEERING

2023/2024

DECLARATION

I hereby, declared this report entitled “Design and Development of Crosslinker Module for Three-Dimensional Bioprinting of Hydrogels” is the result of my own research except as cited in references.

Signature : ……

Author’s Name : MUAMMAR GHADDAFI BIN MOHD ALI

Date : 11 July 2024

APPROVAL

This report is submitted to the Faculty of Industrial and Manufacturing Technology And Engineering of Universiti Teknikal Malaysia Melaka as a partial fulfilment of the requirement for Degree of Manufacturing Engineering (Engineering Design) (Hons). The member of the supervisory committee is as follow:

(DR. MASNI AZIAN BINTI AKIAH)
DR. MASNI-AZIAN AKIAH
Pensyarah Kanan
Fakulti Teknologi dan Kejuruteraan Industri dan Pembuatan
Universiti Teknikal Malaysia Melaka

ABSTRAK

Pencetakan bio tiga dimensi (3D) telah muncul sebagai teknologi yang menjanjikan untuk fabrikasi pembinaan tisu kompleks dalam kejuruteraan tisu dan perubatan regeneratif. Penggunaan hidrogel sebagai bioink untuk pencetakan bio 3D perancah telah mendapat perhatian yang ketara kerana keupayaannya untuk menyediakan persekitaran mikro yang kondusif untuk pertumbuhan sel dan pembentukan tisu. Ketegaran struktur perancah hidrogel sangat bergantung pada proses penghubung silang yang memerlukan larutan berasaskan gelatin untuk bersentuhan dengan larutan berasaskan kalsium. Walau bagaimanapun, pendekatan semasa menggunakan mandian sokongan berasaskan kalsium didapati tidak berkesan dari segi penyediaan mandian dan pempolimeran semasa pencetakan. Oleh itu, projek ini bertujuan untuk mereka bentuk dan membangunkan modul penyambung silang baru untuk pemendapan terus kalsium klorida pada lapisan bioink yang dicetak untuk membolehkan penghubung silang hidrogel yang tepat dan terkawal semasa proses bioprinting. Reka bentuk konsep modul pemaut silang telah direka dengan menggunakan konsep kaedah penyemperitan picagari berganda. Selepas itu, pengoptimuman parameter silang silang dilakukan dengan mengambil kira 7 parameter termasuk bentuk muncung, diameter muncung, ketinggian lapisan, kelajuan cetakan, kadar suapan, suhu dan kepekatan kalsium klorida, didapati bahawa bentuk kon, diameter muncung 14-tolok, 0.8 ketinggian lapisan mm, kelajuan cetakan 3 mm/s, kadar suapan 300 mm/s, suhu 15°C dan kepekatan CaCl 40% adalah parameter yang dioptimumkan. Keberkesanan modul pemaut silang yang dinilai dengan menilai struktur mikro hidrogel yang dicetak menggunakan Pembesaran Mikroskop Optik menunjukkan bahawa sampel yang dicetak menggunakan modul pemaut silang mempamerkan keliangan yang diperlukan untuk pertumbuhan sel. Penemuan daripada kajian ini menyumbang kepada kemajuan teknologi pencetakan bio ke arah merevolusikan industri penjagaan kesihatan.

ABSTRACT

Three-dimensional (3D) bioprinting has emerged as a promising technology for the fabrication of complex tissue constructs in tissue engineering and regenerative medicine. The use of hydrogels as bioinks for 3D bioprinting of scaffold has gained significant attention due to their ability to provide a conducive microenvironment for cell growth and tissue formation. The structural rigidity of the hydrogel scaffold is highly dependent on the crosslinking process which requires the gelatine-based solution to be in contact with a calcium-based solution. However, the current approach of using a calcium-based support bath was found to be ineffective in terms of its bath preparation and polymerization during printing. Therefore, this project aims to design and develop a novel crosslinker module for the direct deposition of calcium chloride onto the printed bioink layer to enable the precise and controlled crosslinking of hydrogels during the bioprinting process. The conceptual design of the crosslinker module was fabricated by applying the concept of double syringe extrusion method. Afterwards, the optimization of the crosslinking parameters was performed by considering 7 parameter including nozzle shape, nozzle diameter, layer height, print speed, feed rate, temperature and calcium chloride concentration, it was found that conical shape, 14-gauge nozzle diameter, 0.8 mm layer height, 3 mm/s print speed, 300 mm/s feed rate, 15°C temperature and 40% CaCl concentration was the optimized parameter. The effectiveness of the crosslinker module evaluated by assessing their microstructural of the printed hydrogel using Optical Microscope Magnification shows that the sample printed using the crosslinker module exhibit porosity which are necessary for cell growth. The findings from this study contributes to the advancement of bioprinting technology toward revolutionizing the health care industry.

DEDICATION

This research study is dedicated to:

My beloved parents;

Mohd Ali Bin Mat Nur

Diana Binti Baco

My respected supervisor,

Dr. Masni Azian Binti Akiah

My siblings and friends,

For their continuous support, motivation, direction, generosity, and moral encouragement throughout this study. I am truly grateful, and may Allah bestow His blessings upon all of us consistently. Thank you.

ACKNOWLEDGEMENT

Bismillahirrahmanirrahim, In the name of Allah, the most beneficent, the most merciful

All gratitude is due to Allah, The Almighty, for graciously bestowing His blessings, health, patience, wisdom, and guidance upon me throughout the course of my research.

I extend my sincere thanks and appreciation to Dr. Masni Azian Binti Akiah, my supervisor, for her invaluable guidance, teaching, and unwavering support, providing me with strength, direction, and emotional encouragement during the completion of my final year project. My heartfelt gratitude goes to my cherished family for their prayers and moral support, instrumental in helping me overcome the challenges and obstacles that surfaced throughout my academic journey.

I express my gratitude to the UTeM management, especially the Faculty of Industrial and Manufacturing Technology and Engineering, for affording me the opportunity to acquire valuable experience and knowledge throughout the research process.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

3D	-	Three Dimensional
CAD	-	Computer Aided Design
UV	-	Ultraviolet
DLP	-	Digital Light Processing
HA	-	Hyaluronic Acid
PLC	-	Polycaprolactone
PLCA	-	Poly(lactic-co-glycolic acid)
PEG	-	Polyethylene Glycol
ECM	-	Extracellular Matrix
HACA	-	catechol-HA
HaCaTs	-	Keratinocytes
TA	-	Tentalum
GEL-MA	-	Gelatin Methacryloyl
CaCl	-	Calcium Chloride
POI	-	Optimization Index
SEM	-	Scanning Electron Microscopy
DC	-	Direct Current
IoT	-	Internet of Thing
TPM	-	Target Performance Measure
NPM	-	Noise Performance Measure
ASTM	-	American Society for Testing and Materials

LIST OF SYMBOLS

Ga	-	Gauge
mm	-	Millilitre
µm	-	Micrometre
%	-	Percent
mPa·s	-	Millipascal Second
w/v	-	Weight per Volume
m/s	-	Mitre per Second
mm/s	-	Millilitre per Second
mm/min	-	Millilitre per Minute
°C	-	Degree Celcius
kPa	-	Kilopascal

CHAPTER 1
INTRODUCTION

1.1 Background of Study

Three-dimensional bioprinting using hydrogels is a promising technology for tissue engineering and regenerative medicine. The bioprinting process applied similar 3D printing mechanism whereby it involves joining material to build up 3D design from CAD data using layer upon layer material deposition. Hydrogels in particular is a biocompatible material that can be used as cell-laden materials for bioprinting (He et al., 2016). They mimic the physical and biochemical properties of natural tissue and have become the preferred bioink for 3D bioprinting applications (Enrique Mancha Sánchez et al., 2020). However, challenges persist in achieving optimal mechanical and biological properties in the printed hydrogel structures. One critical aspect is the need for effective crosslinking methods to stabilize these structures. Since the hydrogel state before print is liquid thus there is a need for it to be in contact with a crosslinker solution to solidify the liquid into gel state. This process is referred as crosslinking.

Recognizing the deficiencies in current crosslinking methods, there is a pressing need for a specialized crosslinker module tailored to hydrogel bioprinting applications. Crosslinking is a process that stabilizes hydrogels by creating a network of polymer chains through covalent or ionic bonding. Hydrogels possess the ability to form scaffolds with a porous structure, crucial for facilitating cell attachment and growth (Amin GhavamiNejad et al., 2020). Consequently, this study aims to bridge this gap by conceptualizing and creating an innovative crosslinker module specifically utilizing calcium chloride. Calcium chloride emerges as a promising candidate owing to its distinctive properties, offering potential advantages in achieving precise and controlled crosslinking within the hydrogel matrix. The rationale for prioritizing calcium chloride as the primary focus lies in its capacity to overcome the limitations associated with conventional crosslinkers.

1.2 Problem Statement

The use of calcium-based bath support bath as the crosslinker medium in current bioprinting is not effective in holding the 3D structure during material deposition. There is potential with direct deposition of the crosslinker material, but the module design is still under development. The effectiveness of direct crosslinker depositor needs to be investigated. Designing and developing a crosslinker module for three-dimensional bioprinting of hydrogels with calcium chloride involves addressing the challenge of maintaining shape fidelity of 3D bioprinted scaffolds with soft biomaterials (Nelson et al., 2021). The crosslinking process is a key procedure that significantly influences the mechanical and physicochemical characteristics of the bioprinted constructs (Amin GhavamiNejad et al., 2020).

Achieving optimal crosslinking is crucial for the mechanical and structural integrity of bioprinted constructs. Existing bioprinters often struggle with the slow crosslinking kinetics of calcium chloride, resulting in uneven distribution and insufficient crosslinking (Katarzyna Bialik-Was et al., 2021). This leads to variations in the stiffness and structural robustness of the printed tissues. These inconsistencies not only hinder the reproducibility of bioprinted structures but can also compromise the functionality and viability of encapsulated cells.

In addition, maintaining both the printability of hydrogels and high cell viability poses a challenge in bioprinting. The delicate balance between achieving optimal printing fidelity and preserving cell viability becomes apparent, particularly when using calcium chloride for crosslinking (Saman Naghieh & Chen, 2021). High concentrations or crosslink densities may be necessary for successful printing, but this could impede cell migration and compromise overall cell health. Conversely, lower concentrations may enhance cell viability but might result in poor printability. This challenge underscores the need for a comprehensive solution that optimizes both printability and cell viability.

Lastly, consistent production of bioprinted hydrogel constructs with the desired mechanical properties and structural integrity is a persistent challenge. Bioprinters, especially when employing calcium chloride-based crosslinking, may struggle to achieve uniform crosslinking throughout the printed structure (Al‐Sabah et al., 2019). Variations in crosslinking efficiency can lead to inconsistencies in the mechanical strength and stability of the printed tissues.

Based on the problem stated, there is a need to design and develop a specialized Crosslinker Module in bioprinter to improve the polymerization and structural integrity during printing of 3-dimensional scaffold.

1.3 Objectives

i. To device the crosslinking module for hydrogel polymerization on existing bioprinter (GelPrint Pro)
ii. To optimize the bioprinting parameter with crosslinker module.
iii. To investigate the structural characteristics of the scaffolds built with the crosslinker module.

1.4 Scope

This project analysis focuses on the crosslinking process and mechanism of the crosslinker module.

I. Research is only limited to crosslinking strategies for hydrogel polymerization.
II. The development of crosslinking module will be based on purely direct input of the previous Gel-Print Pro developer. Hence, the design requirement will not cover any survey from other bioprinter user as this this product is a relatively new model which may not have been publicly commercial yet for the use of the public.
III. Bio-printing will be conducted on a customized hydrogel extruder connected to Snapmaker 3D Printer (GelPrint Pro).
IV. Microstructural properties shall be investigated under Scanning Electron Microscopy SEM.
V. Bioink material will be based on alginate-based materials

1.5 Summary

This study focuses on the design and development of a crosslinker module for 3D bioprinting of hydrogels with calcium chloride. Hydrogels are biocompatible materials that can be used as cell-laden materials for bioprinting, mimicking the physical and biochemical

properties of natural tissues. Crosslinking is a key procedure that significantly influences the mechanical and physicochemical characteristics of the bioprinted constructs. The crosslinking strategy which involves the use of calcium chloride bonds between polymer chains by exploring how varying concentrations of calcium chloride or modifications in the crosslinking process may impact the final bioprinted structures would be valuable. Hence, this study seeks to contribute to the existing knowledge by addressing these identified gaps, delving to device the crosslinking module for hydrogel polymerization on existing bioprinter (GelPrint Pro) by optimize the bioprinting process with crosslinker module and investigate the structural characteristics of the scaffolds built with the crosslinker module.

CHAPTER 2
LITERATURE REVIEW

2.0 Introduction to crosslinking in bioprinting

In the context of 3D bioprinting, the role played by crosslinking in ensuring the stability of printed structures is indispensable. This process facilitates the printing of a liquid, cell-laden "bioink," which is subsequently crosslinked through the formation of a chemical or physical network. The outcome is a set of constructs that possess desirable mechanical properties, cytocompatibility, stability, and sustainability (Knowlton et al., 2017). Notably, crosslinking is a pivotal step that significantly shapes the mechanical and physicochemical characteristics of bioprinted constructs (Amin GhavamiNejad et al., 2020). However, a delicate balance exists between the degree of crosslinking and the resultant structure, with insufficient crosslinking potentially compromising structural integrity and excessive crosslinking affecting printability.

The paramount importance of crosslinking in achieving both mechanical strength and structural integrity is evident in its direct influence on the stability and printability of bioinks, as well as the mechanical behavior of the printed scaffolds (Amin GhavamiNejad et al., 2020). Bioprinting commonly employs various crosslinking methods, encompassing both chemical and physical approaches. Figure 1 show both mechanisms of physical and chemical crosslinking strategies. Chemical methods, exemplified by photocrosslinking, are widely preferred due to their tunability and stability that established through irreversible covalent bonding between polymeric chains. However, precise control of crosslinking kinetics is essential to prevent any obstruction of the printer nozzle. Additionally, physical crosslinking involves secondary forces such as ionic interactions, hydrophobic interactions, hydrogen bonding, or van der Waals forces. Hydrogels that formed through physical crosslinking typically exhibit mechanical weakness but offer a more cell-friendly environment compared to hydrogels crosslinked through chemical methods (Zennifer et al., 2022).

Researchers are also actively investigating dual crosslinking mechanisms as an innovative strategy to preserve the microenvironment of the structure, facilitating the printing of stable and flexible structures (Sriya Yeleswarapu et al., 2023). As an illustration, a strategy involves employing ionic crosslinking for the external layers of the printed scaffold and subsequently utilizing UV crosslinking for the internal layers. Demonstrating efficacy, dual crosslinking mechanisms have proven to enhance the mechanical properties of printed

structures while preserving the microenvironment, enabling the fabrication of stable and flexible structures (Zennifer et al.,2022).

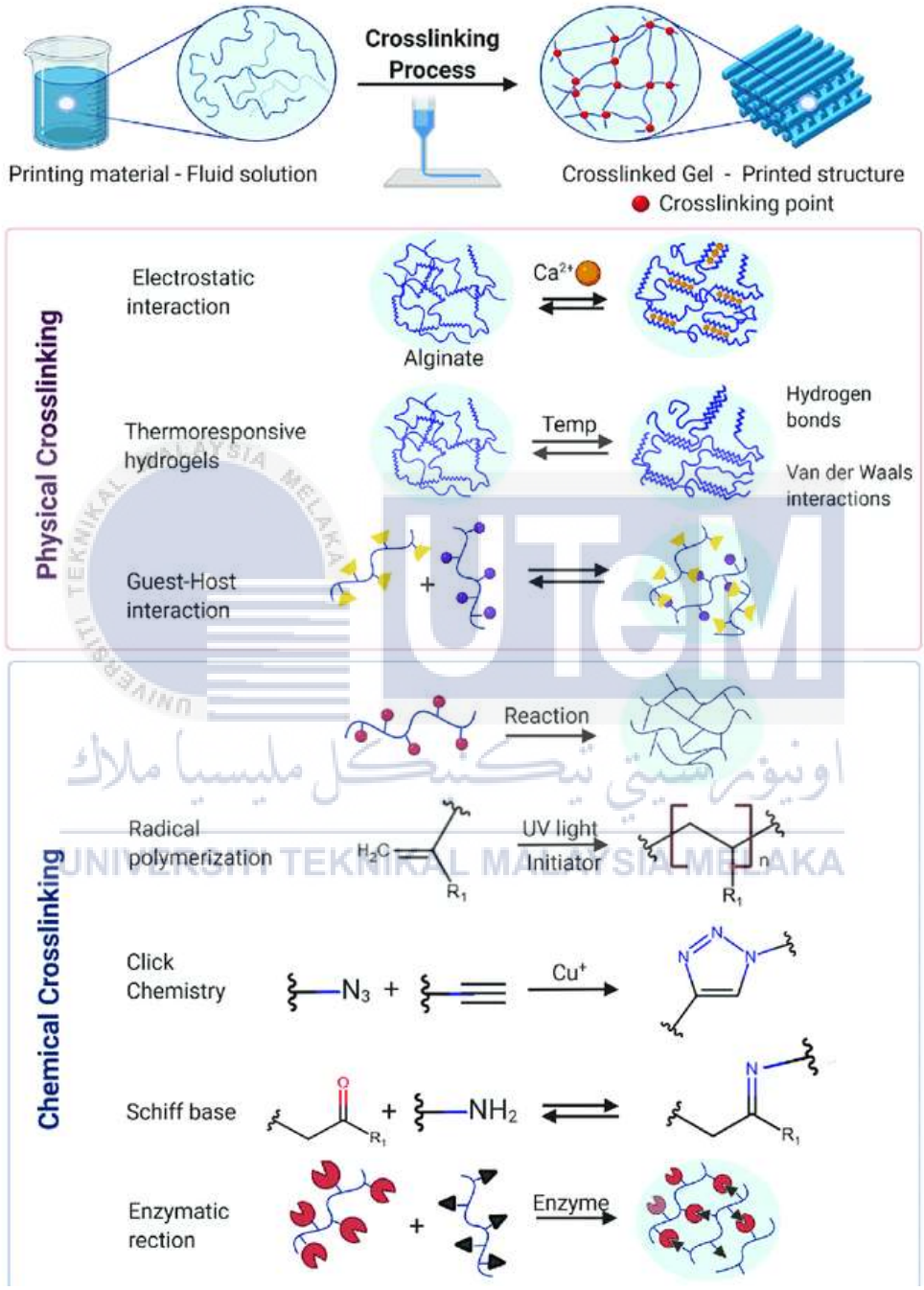


Figure 1: Mechanism of physical and chemical crosslinking (Mabel Barreiro Carpio et al., 2021)

2.1 Bioprinting Technologies and crosslinking

2.1.1 Bioprinting techniques and applications in tissue engineering

Bioprinting technologies have emerged as a formidable tool in the domain of tissue engineering, facilitating the construction of intricate tissue and organ structures. These technologies enable the development of complex and functional tissue architectures using biocompatible materials. There are four main bioprinting techniques that used in tissue engineering, each employing distinct methodologies to fabricate intricate three-dimensional biological structures (Saini et al., 2021; Agarwal et al., 2020).

Droplet-based bioprinting is a technique that operates on the principle of precision deposition, where small droplets of bioink are systematically dispensed through a nozzle or printer head (Wu et al., 2023). The process is driven by mechanisms like thermal, piezoelectric, or pneumatic forces, allowing for the meticulous layer-by-layer construction of the desired biological structure as shown in Figure 2. This method excels in achieving high cell viability due to the gentle formation of droplets, enabling the creation of intricate and cell-laden structures. The controlled droplet deposition ensures that the delicate nature of biological components is preserved throughout the printing process, making it particularly advantageous for applications requiring the intricate arrangement of living cells, such as the construction of tissues with intricate cellular patterns or the development of organoids for detailed biological studies (Kotlarz et al., 2022).

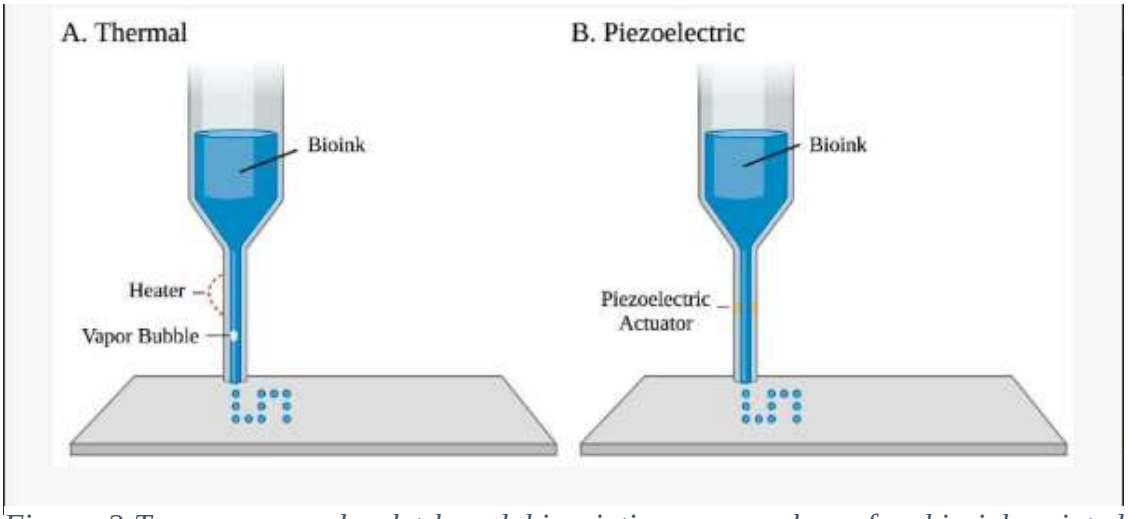


Figure 2:Two common droplet-based bioprinting approaches of a bio-ink printed through an ejector. (A) Thermal-based bioprinting uses localized heating to eject ink droplets along with vapor bubble formation. (B) Piezoelectric-based bioprinting involves an elect.

In laser-assisted bioprinting, a laser is employed to precisely target and transfer bioink to a substrate, resulting in the creation of intricate patterns. The bioink is initially coated on a donor slide, and the laser induces pressure waves, propelling bioink droplets onto a receiving substrate with remarkable precision as shown in Figure 3. This method is characterized by its ability to deposit small volumes of bioink, facilitating the formation of fine structures with varying cell types. Laser-assisted bioprinting thus stands out for its high precision and making it particularly relevant for applications where intricate tissue architectures need to be replicated, such as in the production of artificial organs or tissues with complex cellular compositions (Wu et al., 2023).

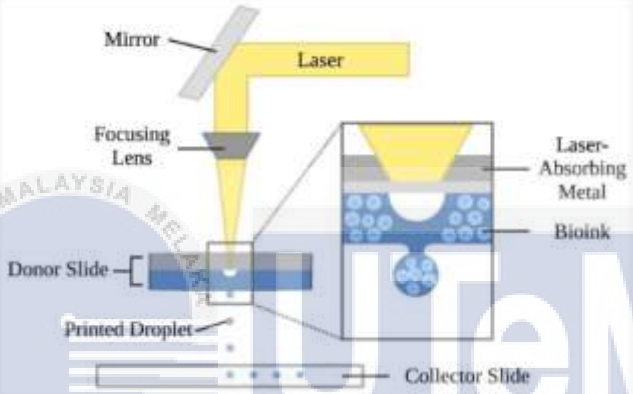


Figure 3:Mechanism of the laser-assisted bioprinting technique, (Wu et al., 2023).

Stereolithography and Digital Light Processing (DLP) Bioprinting leverage light to selectively solidify layers of photosensitive bioink, thereby forming intricate 3D structures (Saeedeh Vanaei et al., 2021). In Stereolithography, a laser serves as the light source, while DLP employs a digital light projector. Both methods as shown in Figure 4, involve the curing of specific areas within a liquid resin containing cells, resulting in high-resolution printed structures. This approach excels in creating detailed structures with a variety of materials, making it suitable in the fabrication of intricately designed tissue constructs, where precision and accuracy are paramount. Examples include the production of customized implants, tissues with specific geometries, or intricate microstructures for biological (Li et al., 2023)

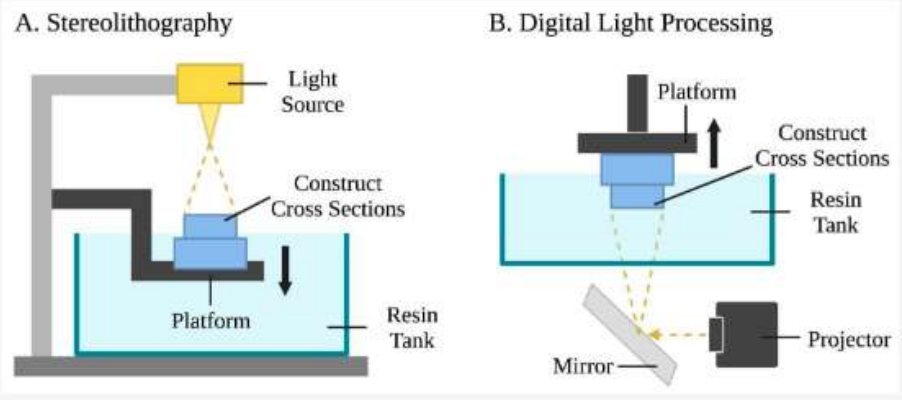


Figure 4:Schematic representations of stereolithography and digital light processing bioprinting. (A) Stereolithography uses a light source (B) Digital light processing uses a light projector and mirror device that reflects the incoming light, (Wu et al., 2023).

Finally, extrusion-based bioprinting utilizes either pneumatic or mechanical forces to extrude bioink through a nozzle, depositing it layer by layer to construct the desired structure as shown in Figure 5. This method involves a computer-controlled system to direct the nozzle in the x-y-z directions ensures the precise placement of the bioink, allowing for the creation of intricate tissue architectures (Rider et al., 2018). One of its primary advantages lies in its versatility, as it is compatible with a wide range of biomaterials, making it suitable for various tissue types and diverse applications within the field of bioprinting (Agarwal et al., 2020). Diverse bioprinting techniques have been devised and applied across the life sciences spectrum, from investigating cellular mechanisms to fabricating tissues and organs for implantation, encompassing structures like heart valves, myocardial tissue, trachea, and blood vessels (Papaioannou et al., 2019)

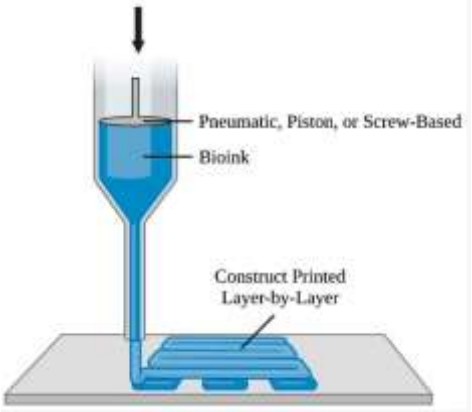


Figure 5:Diagram of the extrusion-based bioprinting approach, (Wu et al., 2023).

2.1.2 Impact of crosslinking on 3D printing

The impact of crosslinking in 3D bioprinting is crucial, especially in addressing the challenges posed by the limited regenerative capacity of adult human organs. Tissue or organ transplantation, the conventional treatment for damage resulting from injury, disease, or surgery, faces constraints due to a shortage of organs, leading to substantial loss of lives during the waiting period for donations. The emergence of 3D bioprinting presents a transformative avenue, allowing for the ex vivo or in situ printing of tissue constructs (Zennifer et al., 2022; Agarwal et al., 2020; Sriya Yeleswarapu et al., 2023).

The impact of crosslinking on 3D printing is profound and encompasses various facets crucial to the production of printed constructs. The mechanical properties of these structures are significantly shaped by crosslinking, as it establishes a stabilizing network, preventing undesired dissolution or deformation under stress (Amin GhavamiNejad et al., 2020). The enhanced mechanical properties contribute to the overall strength, durability, and structural integrity of the printed constructs, making them more resilient and suitable for various applications. Furthermore, appropriate crosslinking ensures the stability and sustainability of living constructs, rendering them well-suited for applications in tissue engineering and biomedical fields, where long-term performance is imperative (Sriya Yeleswarapu et al., 2023). The crosslinked structures exhibit enhanced resistance to degradation over time, promoting their suitability for use in medical implants, prosthetics, or other scenarios where sustained stability is paramount. The influence of crosslinking extends to cellular behavior within the printed structures, affecting vital aspects like viability, proliferation, and differentiation potential (Amin GhavamiNejad et al., 2020). The controlled manipulation of cellular behavior through crosslinking is pivotal for creating tissue constructs with desired biological functions, mimicking natural tissues more closely and advancing the field of regenerative medicine. Striking a delicate balance between the degree of crosslinking and resulting structure is paramount, avoiding issues such as spreading or compromised printability (Zennifer et al., 2022). Too little crosslinking can lead to unwanted spreading of the structure, while excessive crosslinking may hinder printability.

Therefore, crosslinking plays a pivotal role in influencing the mechanical properties, stability, and sustainability of 3D printed constructs, with the choice of method contingent on the specific 3D printing technique employed, necessitating further research for optimized parameters tailored to each approach (Saman Naghieh et al., 2018).

2.1.3 Challenges and limitations of current crosslinking techniques

The existing array of crosslinking techniques within the realm of 3D bioprinting comprises a diverse set of methods, each carrying distinct advantages and inherent challenges The prevalent crosslinking methods in bioprinting encompass in situ crosslinking and photocrosslinking (Galarraga et al., 2019). In situ crosslinking entails solidifying the printed bioink into a stable structure through processes like light exposure or the introduction of calcium ions. Achieving rapid and uniform crosslinking during the in situ bioprinting process poses a challenge primarily due to the dynamic nature of the printing environment (Zennifer et al., 2022). In situ bioprinting involves the deposition of bioink directly at the target site within living tissues, and this process is often subject to inherent movements and deformations. The challenge lies in ensuring that the crosslinking reaction occurs swiftly and uniformly across the printed structure despite these unpredictable movements. Additionally, variations in tissue properties and the complex microenvironment within living tissues further contribute to the difficulty of achieving uniform crosslinking (Mohamadmahdi Samandari et al., 2022). As a result, researchers and engineers working on in situ bioprinting technologies face the ongoing challenge of optimizing parameters and developing strategies to overcome these obstacles and enhance the precision and reliability of the crosslinking process.

On the other hand, photocrosslinking is one of the most widely employed approaches in 3D bioprinting due to its higher crosslinking efficiency and spatiotemporal controllability in developing sustainable tissue constructs (Zennifer et al., 2022). This method utilizes photoinitiators and photo-reactive polymers to enable photoinduced covalent crosslinking, with the intensity of light, exposure time, and area of illumination being critical factors initiates a chemical reaction solidifying the bioink. Zennifer et al., (2022) illustrate using a thick bioink with extrusion-based bioprinting, might need to expose it to light for a longer time during photocrosslinking. This is because a shorter exposure might not let enough light reach the deeper parts of the printed structures, causing improper crosslinking. However, longer exposure could harm the cells and affect the shape of the prints. Additionally, getting rid of leftover materials after crosslinking might be tricky without harming the cells in the prints (Amin GhavamiNejad et al., 2020). This challenge demands deeper insights into the photocrosslinking strategies for successful clinical translation.

2.2 Type of crosslinking agent

2.2.1 Types of materials used in bioprinting

Creating biocompatible, homogeneous, and easily printable biomaterials is crucial for their successful application in 3D bioprinting. Generally, biomaterials refer to natural or synthetic substances used to repair or replace organs within the body. Naturally polymers can be obtained through different methods which are physical, biochemical, or chemical. These polymers have compatibility with biological systems, can retain fluids, and easily dissolve in various solvents like phosphate buffers and cell culture solutions, making them friendly to tissues (Sachdev et al., 2022). Their unique qualities allow for layer-by-layer printing, creating models that closely resemble natural organs when placed in stable environments. An important feature of these polymers is their ability to mimic cells or tissues, undergo growth, maturation, and differentiation when provided with a controlled environment, including normal temperature and proper hydration. However, these activities can be significantly impacted if the surroundings become unstable, such as changes in temperature, dehydration, or alterations in the solvent. Table 1 below shows the material that is commonly used natural polymers in bioprinting.

Table 1: Natural polymer commonly used in bioprinting

Natural Polymer	Description and Characteristics	Application in Bioprinting
Alginate	Alginate is a naturally occurring polysaccharide derived from brown seaweed. It forms hydrogels when crosslinked with divalent cations like calcium ions. Alginate provides structural support and is often used for cell encapsulation.	Commonly used in bioprinting for creating cell-laden constructs.
Gelatin	Gelatin is derived from collagen and is obtained by the partial hydrolysis of animal collagen. It is biocompatible and supports cell adhesion and proliferation. Gelatin is often used as a component in bioinks.	Used as a natural polymer in the bioprinting process, providing support for cell growth and tissue-like structures.
Collagen	Collagen is the main structural protein in the extracellular matrix of various tissues. It offers excellent biocompatibility and is commonly used as	Widely used in bioinks for 3D printing to mimic the natural extracellular matrix

	a bioink component for creating tissues with enhanced cell interaction.	and support cell attachment and differentiation.
Chitosan	Chitosan is derived from chitin, a natural polymer found in the exoskeletons of crustaceans. It possesses antimicrobial properties and is biocompatible. Chitosan-based hydrogels are utilized in bioprinting for tissue engineering.	Applied as a natural polymer in bioprinting applications, particularly for its biocompatibility and antimicrobial features.
Hyaluronic Acid	Hyaluronic Acid (HA) is a glycosaminoglycan present in connective tissues. It has excellent water-retaining properties and is involved in tissue hydration. HA-based bioinks are used for their ability to support cell viability and tissue development.	Used as a natural polymer in bioinks, providing hydration and support for cell viability in the bioprinting of tissue constructs.

Polymers, especially synthetic ones, stand out for their biocompatibility and biodegradability, and often tailored to mimic the properties of natural tissues (Saeedeh Vanaei et al., 2021). In the realm of 3D bioprinting, thermoplastic polymers take center stage, classified into synthetic and natural groups. Synthetic polymers offer superior properties in terms of structure and mechanics, with fewer fabrication limitations. On the other hand, natural polymers, due to their high molecular weight, possess low solubility and high viscosity. Synthetic polymers offer advantages such as precise control over mechanical properties, degradation rates, and functionalization. Typically created through chemical processes, synthetic polymers allow for customization to meet specific requirements. Examples include in Table 2 below polycaprolactone (PCL), poly(lactic-co-glycolic acid) (PLGA), and polyethylene glycol (PEG) are widely used in bioink formulations, providing a versatile platform for creating tissue constructs with controlled properties.

Table 2: Synthetics polymer commonly used in bioprinting.

Synthetic Polymers	Description and Characteristics	Applications in Bioprinting
Polyvinyl Alcohol (PVA)	A water-soluble synthetic polymer with good biocompatibility and suitable for bioink formulation. It exhibits versatility and compatibility with various cell types.	Commonly used in bioinks for 3D printing, providing versatility and compatibility.

Polyethylene Glycol (PEG)	A biocompatible synthetic polymer known for its water solubility. It contributes to bioink formulation, providing a supportive environment for cells.	Employed in bioprinting processes, contributing to bioink formulation and structure.
Polycaprolactone (PCL)	A biodegradable synthetic polymer with a slow degradation rate. It offers mechanical support and stability to printed structures, making it suitable for tissue engineering applications.	Involved in bioprinting applications, offering mechanical support and stability to printed structures.

In this research, hydrogels as the main material crosslink with calcium chloride to printed 3D structure since hydrogels have emerged as a versatile and widely used class of materials in the field of bioprinting, offering unique properties that make them particularly well-suited for the fabrication of three-dimensional (3D) biological constructs (Enrique Mancha Sánchez et al., 2020). The diverse range of materials used in hydrogels, encompassing both natural sources like alginate, gelatin, and collagen and synthetic polymers such as polyethylene glycol and hyaluronic acid, highlights their versatility (Dell et al., 2022). What makes hydrogels particularly attractive is their ability to mimic the extracellular matrix (ECM) of natural tissues, providing a supportive environment for cell growth, proliferation, and differentiation., thereby enhancing the functional restoration of damaged tissues as mentioned by (G. González-Ulloa et al., 2023. Moreover, the mechanical and rheological properties of hydrogels are tunable, allowing for customization to match specific tissue requirements. This tunability, combined with the ability to encapsulate cells, enables the creation of intricate and personalized 3D structures. G. González-Ulloa et al. have extensively explored the mechanical and rheological properties of hydrogels to ensure their printability and stability during the bioprinting process by combining collagen and gelatin in varying ratios and employing a cooling process, hydrogels can be created to exhibit both robust mechanical characteristics linked to collagen and biological properties associated with gelatin simultaneously.

2.2.2 Properties and characteristics of hydrogels suitable for bioprinting

Hydrogels stand out as a versatile and indispensable material in the realm of bioprinting due to their unique properties and characteristics, making them well-suited for applications in tissue engineering and regenerative medicine (Enrique Mancha Sánchez et al., 2020). Hydrogel solutions based on hyaluronic acid (HA) have garnered significant interest as bioinks, presenting promising avenues for advancement in bioprinting applications for skin, nerve tissue, cartilage, and bone. Dell et al., (2022) state that hydrogels exhibit excellent printability and biocompatibility. Printability which is refer to the ability of bioinks to be deposited layer-by-layer into 3D patterns, enabling their use as bioinks in 3D bioprinting processes. The ability to precisely deposit layers of hydrogel-based bioinks facilitates the creation of complex, multi-cellular structures is primarily influenced by their rheological characteristics. In an effort to replicate the full-thickness structure of the skin, Ding et al., (2023) employed a proportional mixture of catechol-HA (HACA) and sodium alginate with calcium chloride, as well as gelatin with horseradish peroxidase and hydrogen peroxide as in Figure 6. After the contact for printing, the two hydrogels underwent chemical crosslinking. Thrombin-free fibrinogen, combined with human dermal fibroblasts, was introduced into the printed scaffolds to induce gelation. Subsequently, the gelatin was removed, and human HaCaTs keratinocytes were seeded onto the scaffolds to proliferate and generate structures resembling the skin. Then, it is proof that hydrogel exhibit excellent printability.

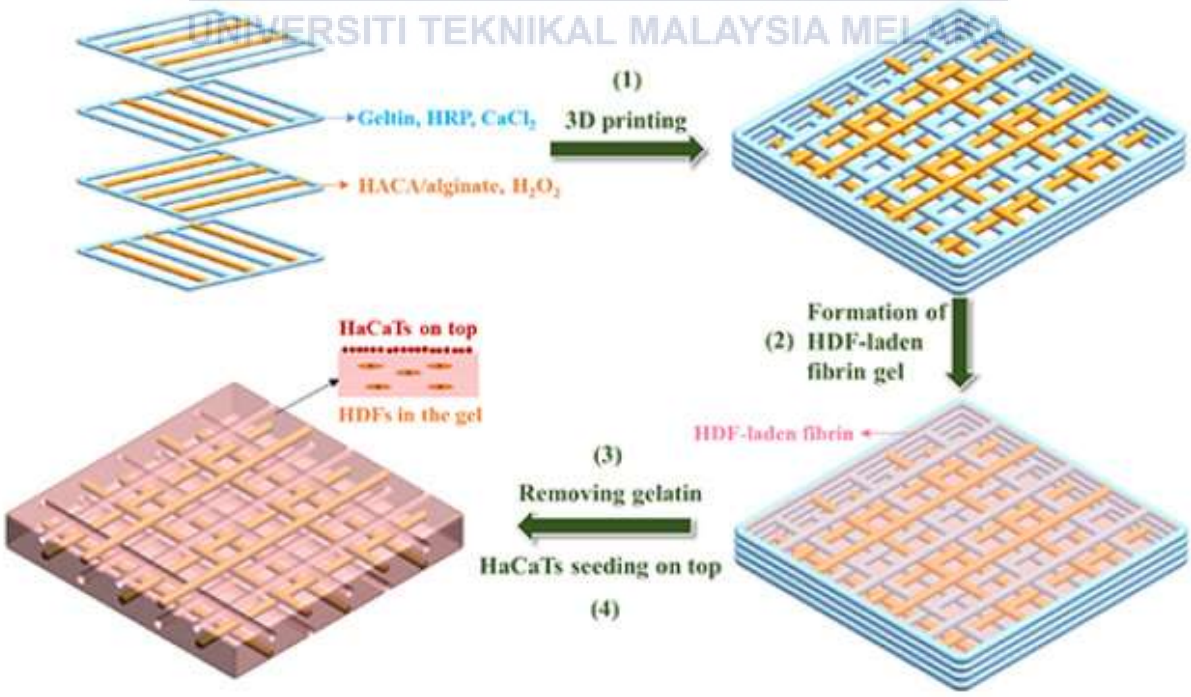


Figure 6:Fabrication of a bilayer cell-laden skin like structure model, (Ding et al., 2023).

On the other hand, biocompatible hydrogels are characterized by their non-toxic nature, fostering a supportive environment for cells and tissues during and after the bioprinting process. These hydrogels induce minimal inflammatory responses, promoting a harmonious integration with living tissues. Hyaluronic acid (HA) stands out as a non-immunogenic natural polymer found in the extracellular matrix of diverse tissues, demonstrating excellent biocompatibility and hydrophilicity. It plays a crucial role in regulating various cell behaviors and tissue functions. However, its intrinsic limitations, such as poor mechanical strength and rapid degradation, render HA unsuitable as a standalone bioink for 3D bioprinting. Ding et al., (2023) have undertaken chemical modifications to HA, incorporating it into a printable hydrogel to create an HA-based hydrogel solution. In contrast to pure HA or other hydrogels, this solution not only retains good biocompatibility and appropriate biodegradability but also imparts the necessary mechanical strength and stiffness essential for effective bioink applications.

Lastly, hydrogels are often composed of shear-thinning materials that have the ability to extrude under high shear stress while maintaining their mechanical properties afterwards (Dell et al., 2022). Certain materials like gelatin and polyethylene glycol (PEG) exhibit a liquid state during the printing process and subsequently transition to a gel-like structure upon extrusion. The higher temperature of the environment, the lower the viscosity, which correlates to less shear stress and less damage to the cells. Dell et al., (2022) describe the enhancement of furfuryl-gelatin can be achieved by incorporating hyaluronic acid, leading to improved viscosity and shear-thinning properties. This modification not only enhances the structural integrity but also contributes to the stiffness of the resulting cross-linked structure. Another method involves the modification of gelatin through the introduction of radically cross-linkable methacryl groups, resulting in gelatin methacryloyl (GEL-MA). This approach stabilizes gelatin, making it suitable for applications in cell inks for bioprinting and various other tissue engineering applications.

2.3 Design and Development of Bioprinting Systems

The field of bioprinting has seen significant development in recent years, with various technologies and systems being employed for different applications. Cyfuse Biomedical has introduced an innovative bioprinting system named Regenova, which operates on the unique "Kenzan" that using droplet-based method (Gu et al., 2020). This technique is renowned for its proficiency in constructing intricate 3D cellular structures. The Regenova bioprinter utilizes the Kenzan method, where cells are precisely layered onto needle arrays, known as Kenzan needles. These needles act as temporary scaffolds, facilitating the assembly of cells into complex 3D configurations. The Kenzan bioprinting system by Cyfuse Biomedical provides a platform for creating finely detailed and functional biological constructs, offering significant promise in the realm of tissue engineering and regenerative medicine.

Furthermore, the EFL-BP8601 and high-precision printer EFL-BP5800 is a photocuring-based bioprinter, and its design is adaptable from existing commercial photocuring-based printers as shown in Figure 7. Gu et al., (2020) state their products offer functional modularization, with independent control modules for pneumatic functions and the option to add a temperature control module. The system is designed for portability and is compatible with standard clean benches. The user-friendly BP6601 is equipped with multi-level schemes and personalized services, catering to both researchers in the early stages of bioprinting platform development and those with advanced needs in regeneration, repair, drug screening, tumor models, personalized medicine, and more. The BP5800 achieves a remarkable printing filament diameter of 3 μm, in contrast to the 100–200 μm printing resolution of conventional 3D printers. This capability allows for the efficient fabrication of high-precision bio-scaffolds, enhancing biocompatibility through significantly improved resolution.

Figure 7:(A) Extrusion-based bioprinter EFL-BP6601; (B) High-precision printer EFL-BP5800 (3 μm resolution), (Gu et al., 2020).

The multifunctional 3D bioprinting system represents a significant advancement in the field of bioprinting technology. Unlike traditional bioprinters, this system integrates various modules that enhance its capabilities, allowing for the creation of more intricate and sophisticated tissue structures. Xu et al., (2022) state that multifunctional 3D bioprinting more versatile and adaptability by an adaptable bioprinting system manufacturing approach was employed in the creation of a microextrusion-based modular 3D bioprinting platform. The platform, encompassing the motion system, nozzle, additive manufacturing platform, cartridges, temperature controller, and UV controller system, has overall dimensions of 300 mm × 300 mm × 300 mm in width, length, and height. This versatile bioprinting platform supports mechanical piston-, pneumatic-, and screw-based microextrusion processes, offering a comprehensive solution for various bioprinting applications as shown in Figure 8 below. In term of adaptability of the multifunctional 3D bioprinter it achieves by employing an integrated software system consisting of an infrared laser sensor and a mechanical position sensor, the system enables the correction of the 3D spatial position of the nozzle tips for all nozzles (the outlet points of the extruded material) that can print a complex geometry. Xu et al., (2022) also developed and produced efficient controls for rapid temperature changes, utilizing semiconductor refrigeration and resistance heating to regulate the temperatures of both the nozzles and the print bed. The schematically for the multifunctional 3D bioprinting system show in Figure 9.

In the foreseeable future, Deo et al., (2020) foresee the advancement of hybrid bioprinting systems with the capability to dispense multiple biomaterials, various cell populations, and diverse biochemical cues, including drugs, nutrients, and growth factors. This progress is expected to bring us closer to achieving the regeneration of whole tissues and organs. Additionally, it will contribute to the evolution of biomanufacturing technologies with in vivo integration, resulting in engineered constructs exhibiting enhanced in-vivo efficacy (Saeedeh Vanaei et al., 2021). The emergence of stimuli-responsive bioprinting strategies is poised to revolutionize healthcare and medicine, introducing dynamic constructs suitable for applications in biosensing, bioactuation, and biorobotics. Deo et al., (2020) also light on the intricate process of 3D bioprinting, providing a comprehensive understanding of its various aspects. It delves into the mechanics governing bioinks and their macroscopic properties, emphasizing the ability to modulate adhesion, degradation, and therapeutic delivery.

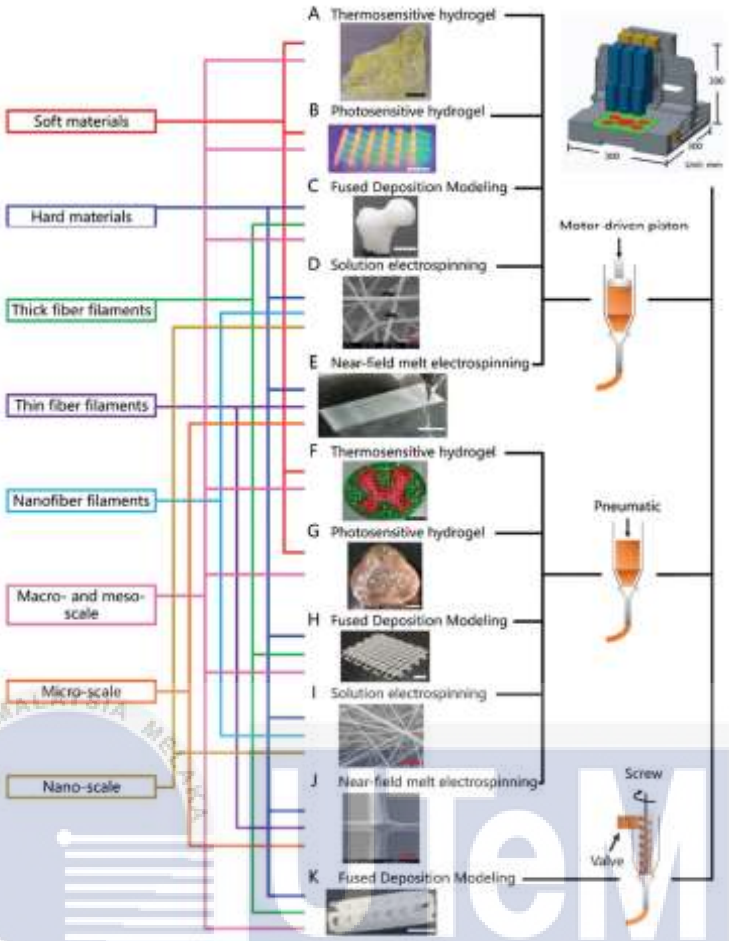


Figure 8:Schematic of the microextrusion-based multifunctional modular 3D bioprinting system, (Xu et al., 2022).

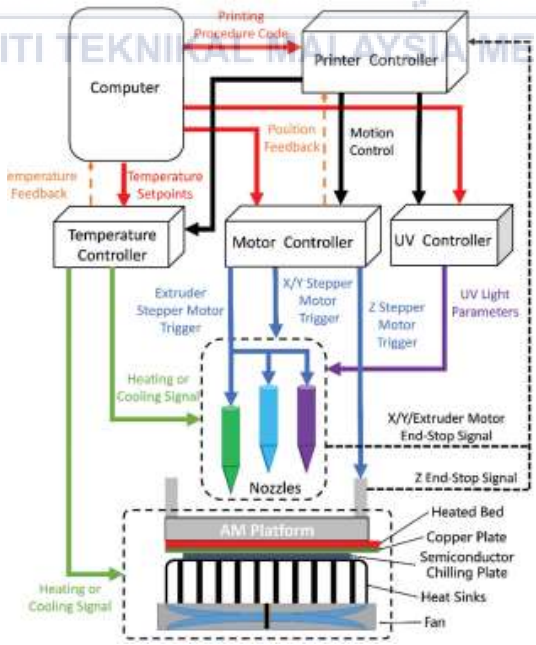


Figure 9:The links between the various units and the direction of flow of the control signals are shown schematically for the multifunctional, (Xu et al., 2022).

2.4 Optimization of Bioprinting Process

2.4.1 Factors affecting the bioprinting process of hydrogel

The bioprinting process of hydrogels is influenced by several factors that can affect the printability and behavior of the printed structures. The viscosity of hydrogels is a crucial parameter influencing their printability. Viscosity, which reflects fluidity, plays a significant role in the extrusion of hydrogels. Higher viscosity levels lead to increased inner pressure during the extrusion process, potentially causing greater cell damage (M. Azad Emin et al., 2021). Iliyana Pepelanova et al., (2018) recommend the use of shear-thinning hydrogels to facilitate filament deposition and achieve high shape fidelity post-printing, particularly under low shear stress conditions. Wei et al., (2023) investigated various alginate concentrations, resulting in viscosities ranging from 13.5 mPa·s (0.5% w/v) to 2,156 mPa·s (4% w/v) that used alginate concentration. As result, considering this range of concentrations that impact hydrogel viscosity, it becomes possible to adjust stiffness to achieve an optimal balance between enhanced shape fidelity (with harder hydrogels) and improved printability (with softer hydrogels).

Furthermore, the curing process, involving cross-linking also essential for ensuring the stability and structural integrity of the printed hydrogel structures. This process contributes to the mechanical properties of hydrogels, including modulus and stiffness, which are influenced by factors like the concentration of photoinitiator and the power of UV irradiation (Dell et al., 2022). Cross-linking refers to the formation of covalent bonds between polymer chains within the hydrogel, creating a three-dimensional network. This network is essential for maintaining the shape and form of the printed structure, preventing it from collapsing or deforming. The choice of cross-linking method, whether chemical, physical, or enzymatic, affects the degree of cross-linking and, consequently, the mechanical properties of the hydrogel (Sang Cheon Lee et al., 2020). Properly controlled cross-linking is crucial for achieving the desired balance between stiffness and flexibility, ensuring that the printed hydrogel structure retains its intended form throughout the bioprinting process. The optimization of the curing process contributes significantly to the success of bioprinting applications, impacting the overall performance and functionality of the printed constructs.

2.4.2 Strategies for optimizing the bioprinting parameters

Optimizing bioprinting parameters is crucial for achieving high-quality and functional bioprinted constructs by developing an optimization index for 3D bioprinting that can significantly enhance the bioprinting process of hydrogels. This index serves as a comprehensive guideline in determining and fine-tuning the optimal print parameters. By establishing an optimization index also can systematically evaluate various parameters such as printing speed, nozzle diameter, and extrusion pressure as shown in Table 3. The objective of the parameter optimization index (POI) is to minimize shear stress exerted on the bioink, and consequently on the encapsulated cells, while ensuring maximum geometric accuracy is achieved (Webb & Doyle, 2017). The study that applied the POI to a mixture of 7% alginate and 8% gelatin, testing the printing under various conditions, including 25, 27, and 30 gauge print nozzles at print speeds ranging from 1 to 6 mm/s and print pressures from 100 to 250 kPa. In total, 72 printing configurations were examined. Webb & Doyle, (2017) indicate that, for specific hydrogel blend, the optimal print is achieved with a 30-gauge nozzle, 100 kPa print pressure, and 4 mm/s print speed. The POI proves to be an intuitive and easily assessable method, demonstrating its potential usefulness across diverse 3D bioprinting research and development applications.

Table 3: Approximate printing parameter effect on printing objectives (Webb & Doyle, 2017)

Parameter	Accuracy (%)	Shear stress (kPa)
Increase print speed	Increase	-
Increase pressure	Decrease	Increase
Increase nozzle diameter	Decrease	Decrease

Selecting the appropriate printing method is a critical aspect of optimizing bioprinting parameters. The choice of printing method significantly influences the outcome of the bioprinting process, affecting aspects such as resolution, accuracy, and the overall quality of the printed structures (Leberfinger et al., 2019). Various bioprinting methods, including extrusion-based, inkjet-based, and laser-assisted techniques, offer different advantages and are suitable for specific applications. For instance, extrusion-based bioprinting is often preferred for its ability to handle a variety of bioinks, including those containing cells and biomaterials, and allows for the deposition of complex structures (He et al., 2016). On the other hand, inkjet-

based bioprinting excels in creating high-resolution patterns, making it suitable for applications requiring intricate details. Laser-assisted bioprinting provides precise control over the deposition of materials and is often used for printing delicate structures. The selection of the right printing method depends on the specific requirements of the intended application, the characteristics of the biomaterials and bioinks, and the desired properties of the final printed constructs. Leberfinger et al., (2019) states that if you need high resolution and intricate structures, laser-assisted printing might be suitable. If versatility and the ability to handle a wide range of hydrogel viscosities are essential, extrusion-based printing could be preferred. It's crucial to consider the nature of the hydrogel, the desired properties of the final construct, and the specific challenges associated with each printing method when making a choice.

2.5 Structural Characterization Techniques

Scanning Electron Microscopy (SEM) is a fundamental technique for investigating the structural characteristics of hydrogel scaffolds in the field of tissue engineering and bioprinting. Scanning electron microscopy (SEM) stands out as the extensively documented approach for examining the microarchitectures of hydrogels. This advanced imaging technique delivers a comprehensive portrayal of hydrogel surfaces, achieving nanoscale precision (Koch & Włodarczyk-Biegun, 2020). From the image, we can see the pore size of the scaffold where more porous will be more suitable for cell growth. Porosity plays a vital role in biomaterial advancement by facilitating the passage of oxygen and nutrients to cells, along with the efficient removal of cellular debris. The porous structure significantly impacts cell migration, tissue integration, and the depth to which cells can penetrate the scaffold. In essence, porosity is paramount for creating an environment that supports cellular activities and overall tissue development. According to Kaczmarek et al., (2020), pores smaller than 5 μm are essential for neovascularization, while those between 5–15 μm impact fibroblast ingrowth. Pores ranging from 20–125 μm encourage the infiltration of adult mammalian cells. Additionally, pores between 40–100 μm facilitate osteoid ingrowth, and those exceeding 500 μm are necessary for fibrovascular tissue growth as show in Figure 10 the combination of sodium alginate and TA provided a porous structure with larger interconnected pores where the crosslinking agent improves the material stability and permitted biomineralization. Hence, meticulous control of scaffold porosity holds the potential to influence biomaterial-tissue interactions.

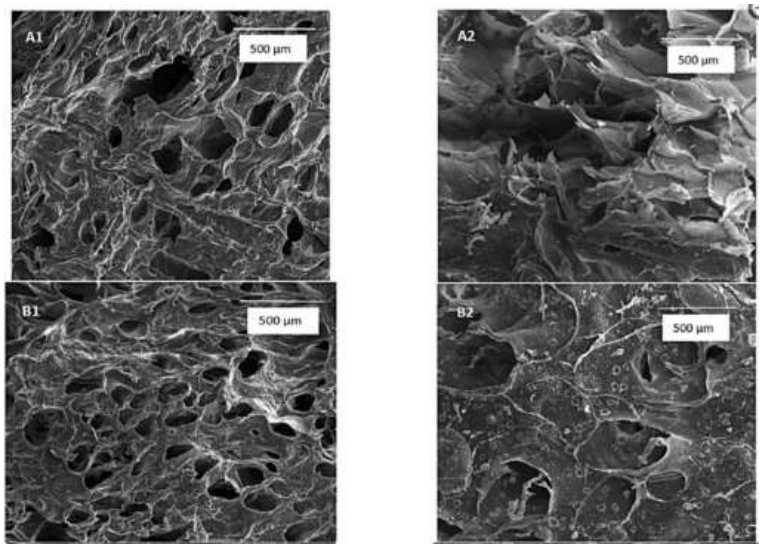


Figure 10: Scanning electron microscopy of hydrogels (A) without TA; (B) 10% TA, Kaczmarek et al., (2020).

Compression testing is a valuable method for analyse both the mechanical and biological characteristics of hydrogel structures. In this testing approach, the hydrogel is subjected to compression forces to understand how it responds under pressure. The mechanical properties, such as stiffness, flexibility, and deformation, can be precisely measured. Additionally, compression testing provides insights into the biological performance of hydrogels, including how they interact with cells and tissues when compressed. An experiment conducted by Nafar Dastgerdi et al., (2021) to maintain consistent contact friction between tests and ensure accurate measurements by changing the paper between each hydrogel specimen. This practice prevented the pores of the paper from previous tests from being filled with hydrogel residue, which could affect subsequent results. The barrelling effect was more pronounced with new pieces of paper, and uneven deformation occurred when using previously used paper that illustrate the cases of applying new and old pieces of paper at the sample machine boundary, with Figure 11 providing additional insights into boundary effects of the radial surface deformation along a vertical line on the specimen surface. The results of new paper smaller boundary compared to old paper indicates the deformation of combining hydrogel slowly deform.

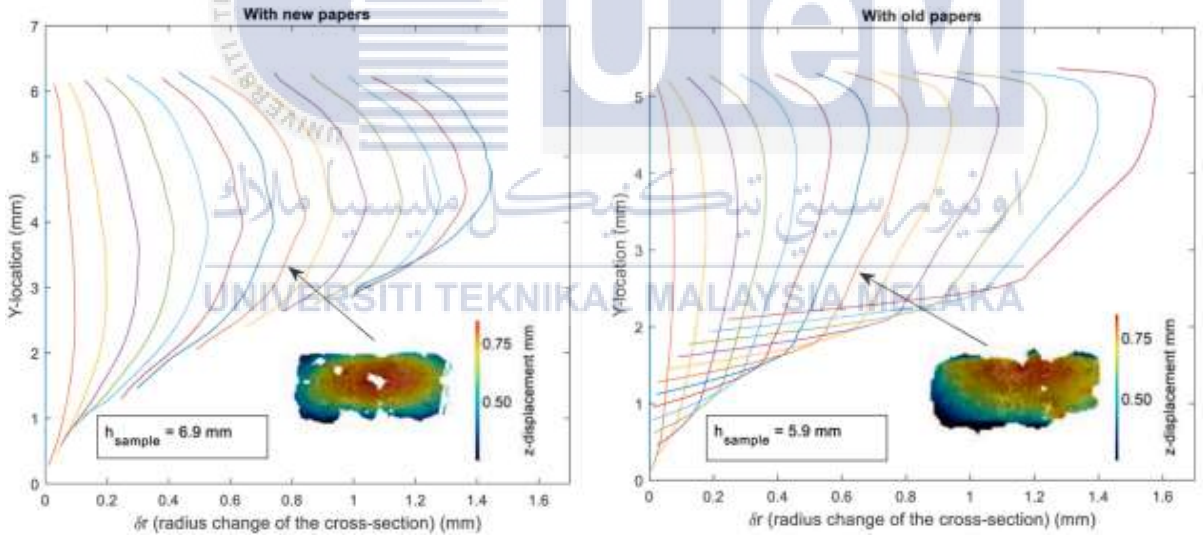


Figure 11: Radial surface deformation for hydrogel specimen during compression testing, Nafar Dastgerdi et al., (2021).

2.6 Crosslinked Hydrogel Scaffolds with Calcium Chloride

In recent research focused on enhancing the mechanical properties of hydrogel scaffolds for bioprinting applications with the use of calcium chloride as a crosslinking agent. Hydrogel scaffolds serve as supportive structures in bioprinting, where the goal is to create three-dimensional biological constructs using living cells. By introducing calcium chloride during the fabrication process, researchers have found that the resulting hydrogel scaffolds exhibit improved mechanical strength and resilience (Al-Sabah et al., 2019). This is crucial in bioprinting as it ensures that the printed structures can better mimic the mechanical characteristics of real tissues in the human body. The choice of calcium chloride as a crosslinking agent is particularly advantageous because it not only enhances the scaffold's strength but also proves to be biocompatible, meaning it doesn't harm the living cells integrated into the printed structures. This is because higher concentration of Ca(II) ions can ensure hydrogel stability, and subsequently, less dehydration (Magdalena Beata Łabowska et al., 2023). In addition, the amount of calcium ions in the hydrogel structure also affects the color and transparency of the resulting hydrogels. The higher degree of cross-linking makes the hydrogel less transparent.

Calcium chloride stands out as an effective and safe option for reinforcing hydrogel scaffolds in bioprinting compared to other crosslinker agent, bringing us closer to the development of robust and functional artificial tissues (Wei et al., 2023) (Rasheed et al., 2020). The research from Zerihun Feyissa et al., (2023) which is double network sodium alginate/chitosan hydrogels were prepared using calcium chloride (CaCl2) and glutaraldehyde as the crosslinking agents by the ionotropic interaction method for controlled metronidazole release. The study delves into the impact of polymer ratios and the quantity of CaCl2, exploring their influence on porosity, gel fraction, and swelling behavior in simulated physiological fluids. As the result, the interaction dynamics between the polymers and CaCl2, resulting in the formation of crosslinked structures, ensuring good stability, and influencing the phase nature and morphology of the hydrogels compared to glutaraldehyde agent. Ongoing studies are likely to further optimize the use of calcium chloride and explore additional techniques to advance the capabilities of bioprinting in tissue engineering and regenerative medicine.

2.7 Conclusion and Research Gap Identification

In conclusion, the current state of research in the field of bioprinting, with a specific focus on crosslinking, reveals a comprehensive exploration of various aspects such as bioprinting technologies, types of crosslinking agents, design and development of bioprinting systems, optimization processes, and structural characterization techniques. The significance of crosslinking in bioprinting is evident in its impact on the 3D printing process, especially in the development of hydrogel scaffolds. The outcome of the research, in situ crosslinking technique and extrusion-based bioprinting has potential and promising result in printing hydrogel scaffold (Papaioannou et al., 2019; Li et al., 2022). However, amidst the existing body of literature, several gaps and areas for further investigation emerge. Firstly, the literature extensively covers the types of materials used in bioprinting and the properties of hydrogels suitable for the process. Still, a more nuanced understanding of how these materials interact with different crosslinking agents, especially with a focus on calcium chloride, remains limited. The specific role of calcium chloride in the crosslinking process and its influence on the structural and mechanical properties of hydrogel scaffolds warrants deeper exploration.

Furthermore, while there is a wealth of information on the challenges and limitations associated with current crosslinking techniques, there is a need for more targeted studies addressing these issues within the context of calcium chloride crosslinked hydrogel scaffolds. This research could shed light on overcoming challenges and optimizing the use of calcium chloride in bioprinting applications by developing the bioprinter module. The optimization of bioprinting processes and parameters is a key aspect, and while general strategies are outlined in the literature, a specific focus on the optimization of calcium chloride crosslinked hydrogel bioprinting is an area that demands more attention (Webb & Doyle, 2017). Exploring how varying concentrations of calcium chloride or modifications in the crosslinking process may impact the final bioprinted structures would be valuable.

In the nutshell, this study seeks to contribute to the existing knowledge by addressing these identified gaps, delving to to design, develop and integrate the crosslinking module for hydrogel polymerization on existing bioprinter (GelPrint Pro) by optimizing the bioprinting process with crosslinker module and investigate the structural characteristics of the scaffolds built with the crosslinker module.

CHAPTER 3
METHODOLOGY

3.0 Introduction

This chapter presents the methodology of the study. The procedures, equipment and software used to perform the study will be mentioned and elaborated to attain the objectives in Chapter 1. Firstly, the method to achieve the objective is to design, develop and integrate the crosslinker module for hydrogel polymerization on existing bioprinter (GelPrintPro) that is effective to allow crosslinking process to happen during bioprinting. Secondly, to optimize the bioprinting process for crosslinker module to determine the printing and crosslinking parameters for achieving the best rheological properties of bioink employed. Lastly, to investigate the structural characteristics of the scaffold, build with the crosslinker module to understand mechanical and structural characteristics of bioinks to ensure sustainability for cell growth medium. The methods and techniques used to meet the objectives will be described in this part of the study.

3.1 Project Flowchart

The study concentrates on assessing the crosslinker module for calcium based crosslinker agent attach to existing Gel-print pro hydrogel plunger and retracting system. Prior to commencing the investigation, it is imperative to optimize the printing parameters to obtain the mechanical and structural characteristics of the scaffolds built with the crosslinker module.

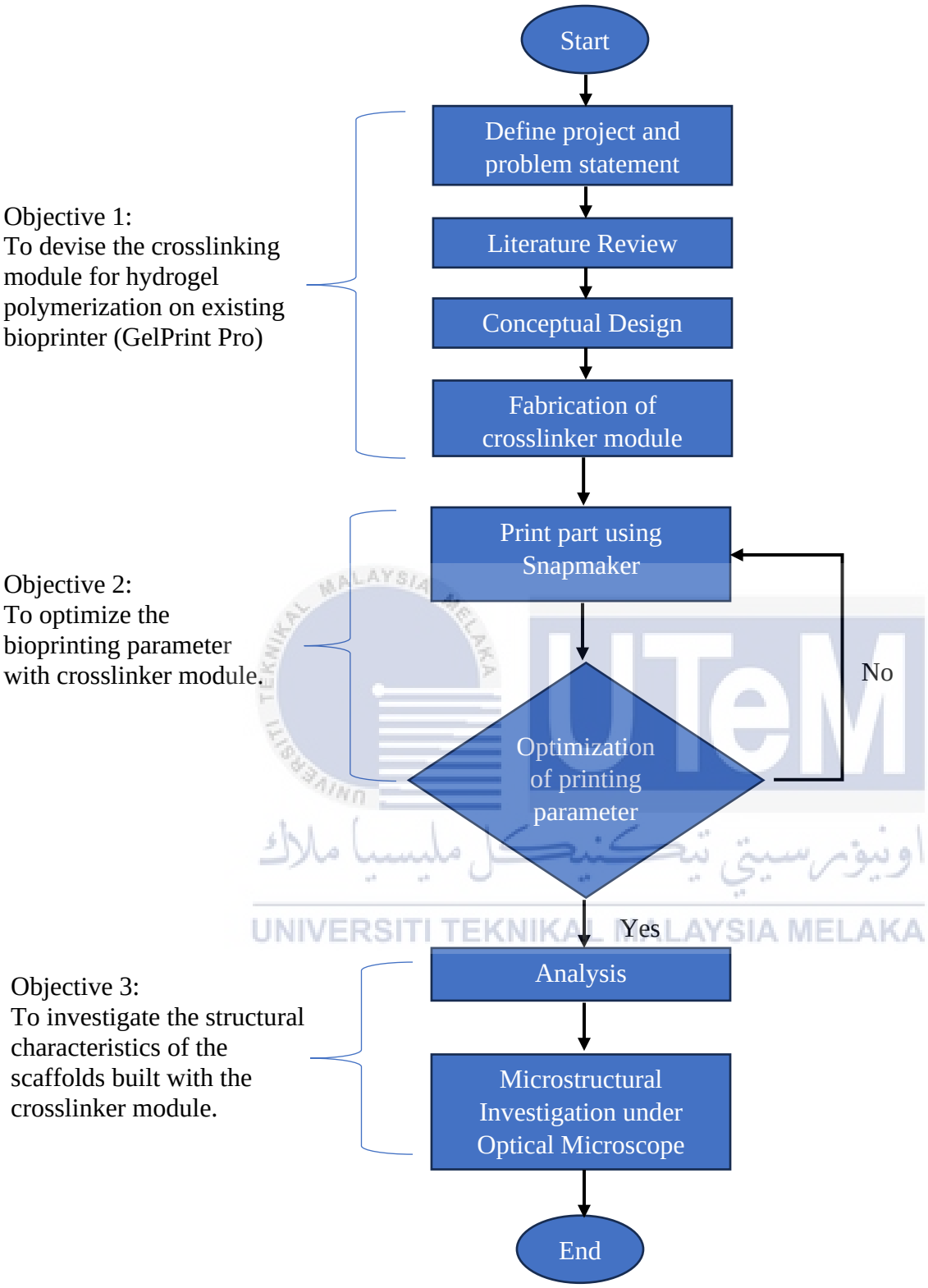


Figure 12: Flowchart of this project

The process flow chart depicted in Figure 12 illustrates the methodology employed in designing and developing a crosslinker module for 3D bioprinting of hydrogels. The initial step of the process involves identifying the background, problem statement, objectives, scope, and research significance of the study. Subsequently, a comprehensive literature review is conducted to crosslinking methods and strategies, optimization of printing parameter, and their structural and mechanical characteristics. From the literature review, the conceptual design of the crosslinker module for calcium chloride (crosslinker agent) are proposed and fabricated. If the crosslinker module unable to print the hydrogel, the crosslinker module will be redesigned.

The next stage of the process involves the optimization of bioprinting parameter for the crosslinker module construct using Gel print Pro Snapmaker by printing multiple hydrogels scaffold. The printed scaffold will visualize in many ways to ensure that the materials meet the desired specifications which are continuous flow, high accuracy and improve structure.

In the event that the materials are deemed acceptable, further testing is conducted, which is micro-structural investigation using optical microscope. Data analysis and reporting are subsequently carried out and compare with previous crosslinker module which is using support bath of calcium-based solution and sample from another researcher on hydrogel.

3.2 Conceptual Design

The conceptual design of the crosslinker module for calcium chloride (crosslinker agent) propose to overcome the problem faces during printing which are unstable crosslinking kinetic, inconsistent production of hydrogel and accuracy of the printer. Based on the research, the best approach in printing the hydrogels scaffold are in-situ crosslinking and extrusion based bioprinting. In situ crosslinking is a technique used in tissue engineering refers to the formation of links or bonds between molecules, creating a more stable and durable structure. In situ means extrude both bioink and calcium chloride at the same time directly during printing the scaffold within the biological environment.

On the other hand, extrusion based bioprinting utilizes versatility, as it is compatible with a wide range of biomaterials, making it suitable for various tissue types and diverse applications within the field of bioprinting. Extrusion-based bioprinting also stands out for its rapid printing speed and impressive resolution capabilities, allowing for the swift and precise

fabrication of intricate and complex structures. It is suitable for running the multiple experiment for optimization of bioprinting parameter due to rapid printing speed.

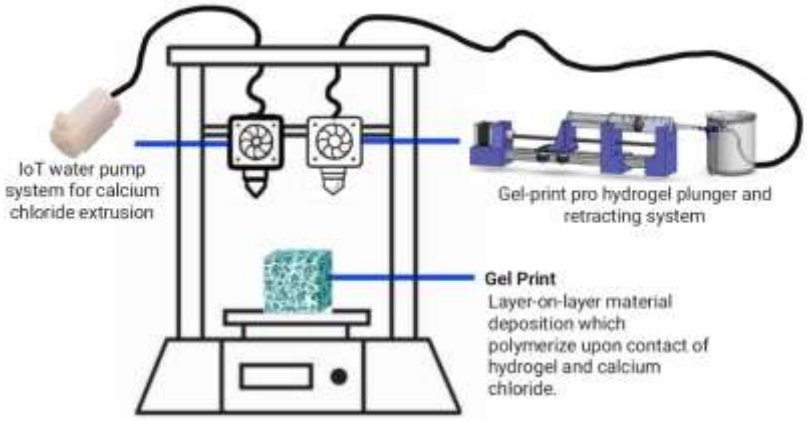


Figure 13: Schematic configuration of the Snapmaker 3D printer

Figure 13 illustrate the schematic configuration of the Snapmaker 3D printer that applying the in-situ crosslinking technique and extrusion based bioprinting. The current printer already builds with hydrogel plunger with retracting system. The calcium chloride needs to redesign from support bath technique because it is ineffective due to tedious preparation and poor hydrogel build-up. The conceptual design for calcium chloride extrusion system will construct using IoT DC water pump. Employing a DC water pump for the extrusion of a calcium-based solution in bioprinting holds potential advantages due to its ability to provide controlled and consistent fluid flow. The conceptual design of calcium chloride extrusion module will be following the mechanism as in the schematic diagram in Figure 14.

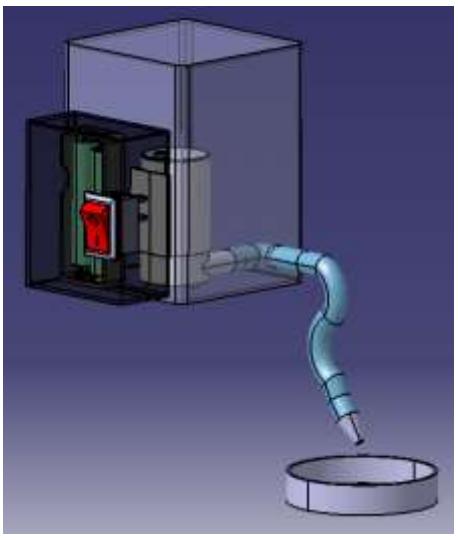


Figure 14: DC Water Pump extruder for calcium chloride (CaCl)

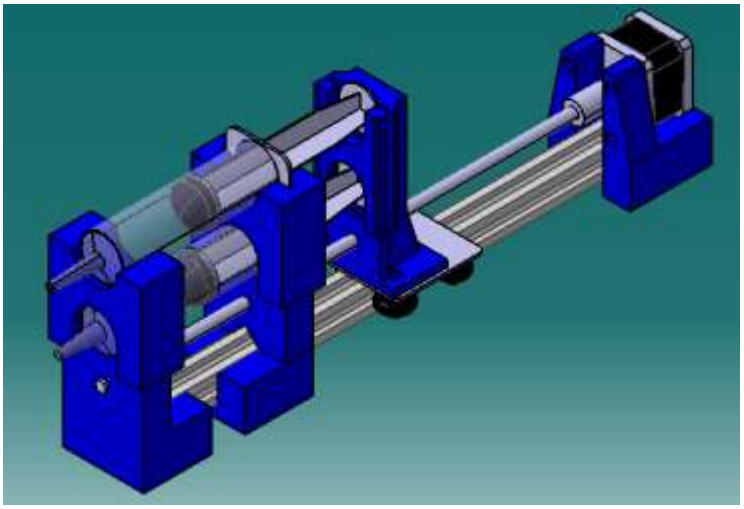


Figure 15: Concept design of double syringe extruder system

The conceptual design of IoT-integrated hydrogel printing system utilizing a DC water pump faces challenges in achieving precise control of extrusion rate and efficient crosslinking, which are high extrusion rate that blows the bioink during printing after fabrication of the crosslinker module. To address these issues, a new concept design, the Double Syringe Extruder System, is proposed as shown in Figure 15. This system employs two syringes, each controlled by stepper motors for precise dispensing: one syringe for the hydrogel precursor and the other for the crosslinking agent. This approach aims to resolve the limitations of the current setup, resulting in a more efficient and scalable hydrogel printing process.

3.2.1 Fabrication of crosslinker module

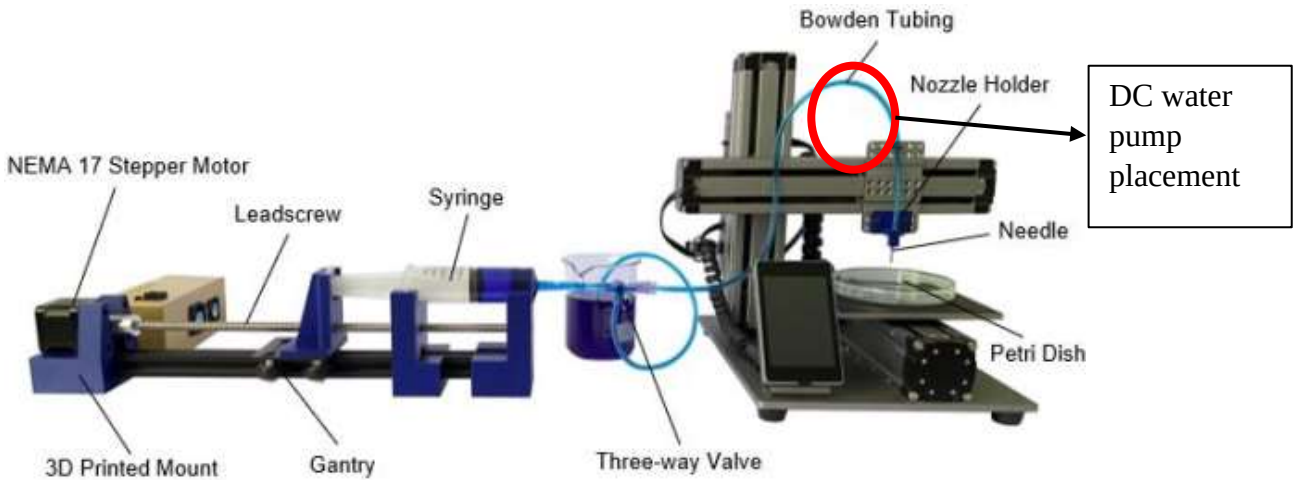


Figure 16: Snapmaker © 3D printer modified with Modular-based Syringe Extruder (MSE) (Ann,A. 2021)

A Snapmaker 3D printer is being used for the 3D bioprinting setup. The thermoplastic extruder on the x-axis carriage of the Snapmaker module was taken apart, and a new nozzle holder was put in. The NEMA 17 stepper motor from the original Snapmaker thermoplastic extruder was used in creating the syringe pump extruder. A 4mm outer diameter tubing was used to connect the extruder nozzle to the x-axis carriage. For customization, a small modification to the G-code was made using the Snapmaker Luban application. The new crosslinker module for the calcium-based solution using IoT DC motor water pump was attached at the red circle mark in Figure 15.

Figure 17: DC water pump Extruder module

The first crosslinker module concept design which is DC water pump integrated with IoT already fabricated as shown in Figure 17. The DC water pump is responsible for managing and controlling the flow of liquids of calcium chloride solution, while IoT integration involves sensors and controllers to control the flow rate during printing. The hydrogel printing mechanism was using the current module as in figure 16. However, this setup faces significant challenges. The DC water pump has fixed extrusion rate where the use of IoT are unable to control the high extrusion rate. Additionally, efficient crosslinking, which is essential for the hydrogel's stability and functionality, is not adequately managed by the current system due to high extrusion rate of crosslinker agent that blow away the hydrogels during printing. The new conceptual design was proposed to overcome the challenge and successfully print the scaffold.

Figure 18: Double syringe extruder system

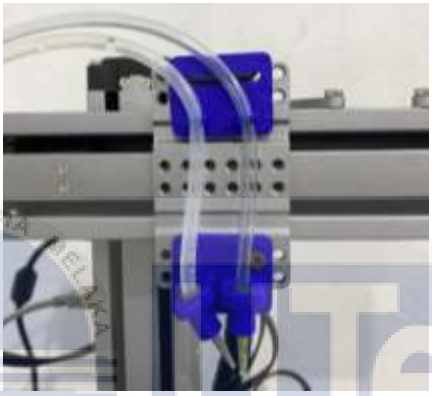


Figure 19: Nozzle holder of the system

The Double Syringe Extruder System for hydrogel printing as shown in Figure 18 consists of several critical components designed to improve precision and efficiency. The system includes two syringes, one for dispensing the hydrogel precursor and the other for the crosslinking agent. Stepper motors provide precise control over the extrusion process, ensuring accurate and synchronized dispensing from both syringes. A nozzle holder as in Figure 19 for the hydrogel and crosslinker uniformly during extrusion, allowing for immediate crosslinking. This system offers several advantages over the existing setup. The stepper motors provide much greater control over the extrusion process compared to DC water pumps, significantly improving precision and able to print the hydrogel scaffold. The system's scalability allows for easy modification to accommodate different types of hydrogels or crosslinkers. The system is then tested and calibrated with various hydrogel and crosslinker formulations to fine-tune the extrusion rates and mixing efficiency.

3.3 Optimization of bioprinting parameters

Robust optimization technique will be used as the design of experiment. The $L_8(2^7)$ orthogonal array will be used to identify the influent of the printing parameter by using specific table to obtain the best printing scaffold of hydrogel. The notation of $L_8(2^7)$ as shown below,

L = Represent the Latin square (notation indicates that the information is an orthogonal array).

8 = Number of row (the number of experiments required)

2 = Number of level (the number of factor level)

7 = Number of column (the number of factors studied)

3.3.1 Bioprinting parameters

A fishbone diagram is like a helpful map for improving bioprinting. It sorts out all the factors that can affect the process, like materials, equipment, environment, personnel, and methods. Figure 16 shows fishbone diagram for factor effecting printed scaffold where the factor is found based on the literature review.

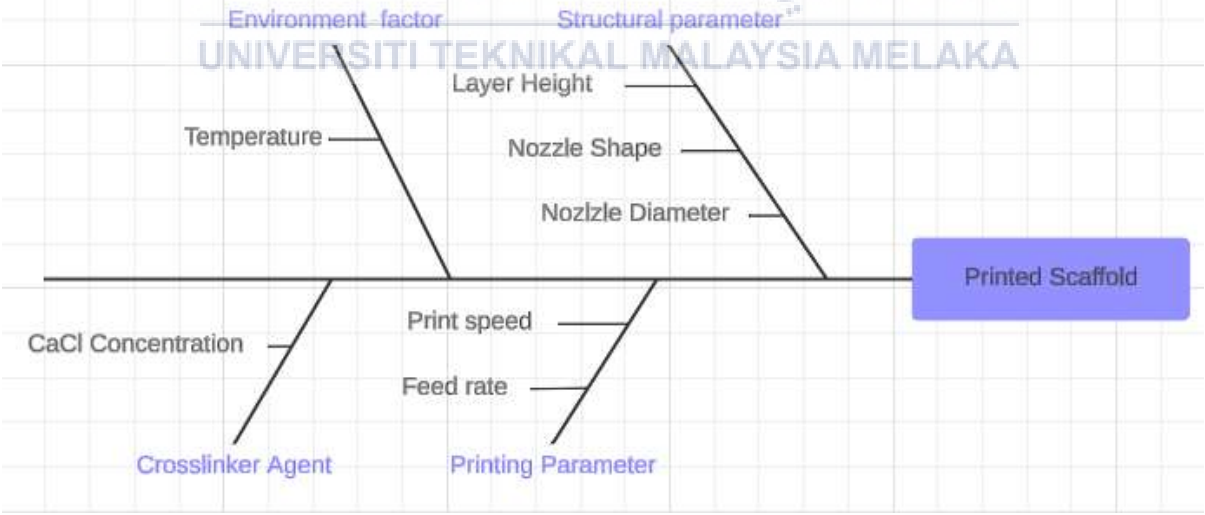


Figure 20: Fishbone diagram for factor effecting printed scaffold.

Table 4: Factor Effecting printed scaffold

No	Factor	Level 1	Level 2
A	Nozzle shape	Cylindrical	Conical
B	Nozzle diameter (ga)	14	16
C	Layer height (mm)	0.8	1.2
D	Print speed (m/s)	2	3
E	Feed rate (mm/s)	240	300
F	Temperature (°C)	30	15
G	CaCl Concentration (%)	20%	40%

The factor that effecting printed scaffold identified from the fishbone diagram and illustrated into Table 4. The value assigned for each factor was obtained from the literature review and previous study using support bath crosslinker module.

3.3.2 Optimization data evaluation

After identifying the most influence parameters, an experimental validation was conducted to obtain the optimum printing quality by running an experiment for 3 specimens of each factor and level with total of 24 printed scaffold. The data was analyse using visual interpretation based on the scoring scale in table 5 and the list level of experiment parameters was run according to table 6.

Table 5: Scoring scale (Masni Azian et al., 2023)

Condition	Example	Score/rank	Description
Under Extrusion		1	Not continuous print Layer thickness < Nozzle diameter
Good Extrusion		2	Continuous print Layer thickness = Nozzle diameter (± 0.1 mm)
Over Extrusion		3	Continuous thickness Layer thickness > Nozzle diameter

Table 6: List level of experiment

Orthogonal Arrays $L_8(2^7)$							
No experiment	A	B	C	D	E	F	G
1	1	1	1	1	1	1	1
2	1	1	1	2	2	2	2
3	1	2	2	1	1	2	2
4	1	2	2	2	2	1	1
5	2	1	2	1	2	1	2
6	2	1	2	2	1	2	1
7	2	2	1	1	2	2	1
8	2	2	1	2	1	1	2

Data was analysed using target performance measure (TPM), essentially a measure of the mean response. It is used to identify control factors that largely affect the mean (and not the variability). These factors are called target control factors. Target control factors was used to adjust the mean response to parameter that effect the printing scaffold quality. The graph was generate using Minitab software and analyse the parameter that affect the printing scaffold quality was analyzed by following the nominal the best ratio.

$$\text{TPM} = \frac{\sum y}{n}$$

y = scoring experiment

n = no of experiment

3.4 Analysis

3.4.1 Microstructural investigation using Optical Microscope

Microstructural investigation of hydrogel using an optical microscope as shown in Figure 20 involves examining the microstructures of bio-ink samples. This process begins with preparing a small hydrogel sample and placing it on a microscope slide, potentially using a coverslip and staining agents for better visibility. Using various objective lenses and proper illumination techniques, the sample is observed to reveal details about the network structure, pore size, and distribution, as well as any inclusions or defects. For this experiment will use 50x magnification with 80 μm. High-resolution images are captured for further analysis, and image analysis software may quantify features such as pore size and network density. This investigation provides insights into the hydrogel's properties and guides further development and optimization, with findings documented in a detailed report that links microstructural characteristics to macroscopic performance. Before doing the analysis, the sample needs to be dry under sunlight for 1 days to evaporate the water insight the sample and breaking it to get the cross-sectional area. The result will compare to the previous study using support bath and study from other researcher on the hydrogels.

Figure 21: Optical Microscope

CHAPTER 4

RESULTS AND DISCUSSION

4.0 Validation of double syringe extrusion system

Validation of the double syringe extruder system in figure 19 involves systematically testing its ability to print different types of hydrogels which are pure bioink (2% alginate), (1% alginate, 1% seaweed) and (2% alginate, 1% seaweed) as in Figure 22,23 and 24 respectively and evaluating the outcomes to ensure that all the hydrogels can be printed using the crosslinker module. The process begins by selecting a range of hydrogel formulations with varying properties and preparing the hydrogel precursor and crosslinker solutions which is calcium chloride. The syringes are loaded, and the stepper motors are calibrated to dispense the solutions simultaneously, ensuring uniform extrusion in the nozzle during printing by following the same G-code for each hydrogel. The crosslinker module are successfully validate as the ability to print different hydrogels and good polymerization achieved as shown in Figure 22,23 and 24.

Figure 22: 2% alginate

Figure 23: 1% alginate, 1% seaweed

Figure 24: 1% alginate, 2% Seaweed

4.1 Optimization of printing parameters

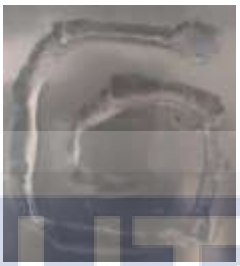
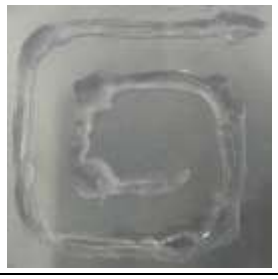
Table 7: List level of printing parameter

Exp No.	Nozzle Shape	Nozzle Diameter (Gauge)	Layer Height (mm)	Print Speed (mm/s)	Feed rate (mm/s)	Temperature (°C)	CaCl Concentration (%)
1	Cylindrical	14	0.8	2	240	30	20
2	Cylindrical	14	0.8	3	300	15	40
3	Cylindrical	16	1.2	2	240	15	40
4	Cylindrical	16	1.2	3	300	30	20
5	Conical	14	1.2	2	300	30	40
6	Conical	14	1.2	3	240	15	20
7	Conical	16	0.8	2	300	15	20
8	Conical	16	0.8	3	240	30	40

The experiments described in Table 7 are designed to systematically investigate the effects of various printing parameters on the quality and performance of printed hydrogels. Using an orthogonal array helps to efficiently explore the parameter space, providing insights into the influence of each parameter while minimizing the number of experiments required. Each experiment in the array is run with three samples with total 27 experiment to achieve optimized printing parameter, the response data and graph by using the pure bioink (2% alginate).

4.1.1 Experimental Result

Table 8: Scoring table

Exp	Sample 1	Sample 2	Sample 3
1			
Scoring	1	2	1
2			
Scoring	3	3	3
3			
Scoring	1	1	1
4			
Scoring	1	1	1
5			

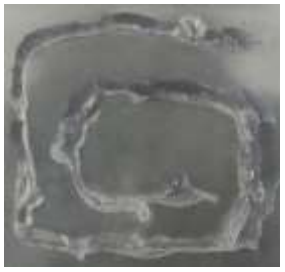
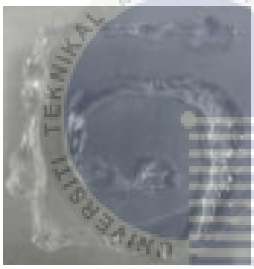
Scoring	2	1	2
6			
Scoring	2	3	1
7			
Scoring	2	3	1
8			
Scoring	3	3	3

Table 8 depicted a visual representation of result obtained from analysing eight experimental with 3 sample according to the list of level of experiment in Table 7. It was observed that some parameter resulted to under extrusion where from 8 experiment 6 experiments had under extrusion score. For good and over extrusion score both have 4 out of 8 experiments. The print quality was usually graded into scoring system of 1: under extrude, 2: good extrusion and 3: over extrude as in scoring scale at Table 4. The average score will calculate for each experiment to be used as final score as in Table 9 for analysing using Minitab.

Table 9: Final score table

Exp No	Sample 1	Sample 2	Sample 3	Average score
1	1	2	1	1.3
2	3	3	3	3

3	1	1	1	1
4	1	1	1	1
5	2	1	2	1.6
6	2	3	1	2
7	2	3	1	2
8	3	3	3	3

4.1.2 Data analysis using Minitab

Table 10: Response table for mean from Minitab software

Response Table for Means

Level	Nozzle Shape	Nozzle Diameter	Layer Height	Print Speed	Feedrate	Temperature	CaCl Percentage
1	1.575	1.975	2.325	1.475	1.825	2.000	1.575
2	2.150	1.750	1.400	2.250	1.900	1.725	2.150
Delta	0.575	0.225	0.925	0.775	0.075	0.275	0.575
Rank	3.5	6	1	2	7	5	3.5

Table 10 above presents an analysis of Total Productive Maintenance (TPM) in relation to various 3D printing parameters, highlighting the impact of each parameter on TPM through a comparison of two levels for each parameter. The most influential factor is the layer height, with a difference of 0.925 between the two levels, indicating it has the highest impact on TPM and thus is ranked first. This suggests that optimizing the layer height could lead to the most significant improvements in the 3D printing process. The print speed follows as the second most influential factor, with a difference of 0.775, suggesting it also plays a crucial role in determining TPM.

Nozzle shape and CaCl concentration both show a moderate impact, with equal differences of 0.575, tying for the fourth rank. This indicates that while these parameters are important, they do not influence TPM as significantly as layer height and print speed. Temperature also shows a moderate impact with a difference of 0.275, ranking third, which suggests that it is a parameter worth considering but not as critical as the top two factors.

Conversely, nozzle diameter and feed rate have minimal impacts on TPM, with differences of 0.225 and 0.075, respectively. This implies that variations in these parameters are less likely to affect the overall productivity and maintenance of the 3D printing process significantly. Therefore, while they should not be neglected, their optimization would yield comparatively smaller improvements.

In summary, this analysis indicates that to enhance TPM in 3D printing, primary focus should be on optimizing layer height and print speed, followed by considerations for nozzle shape, CaCl concentration, and temperature, while nozzle diameter and feed rate can be given lower priority due to their minimal impact.

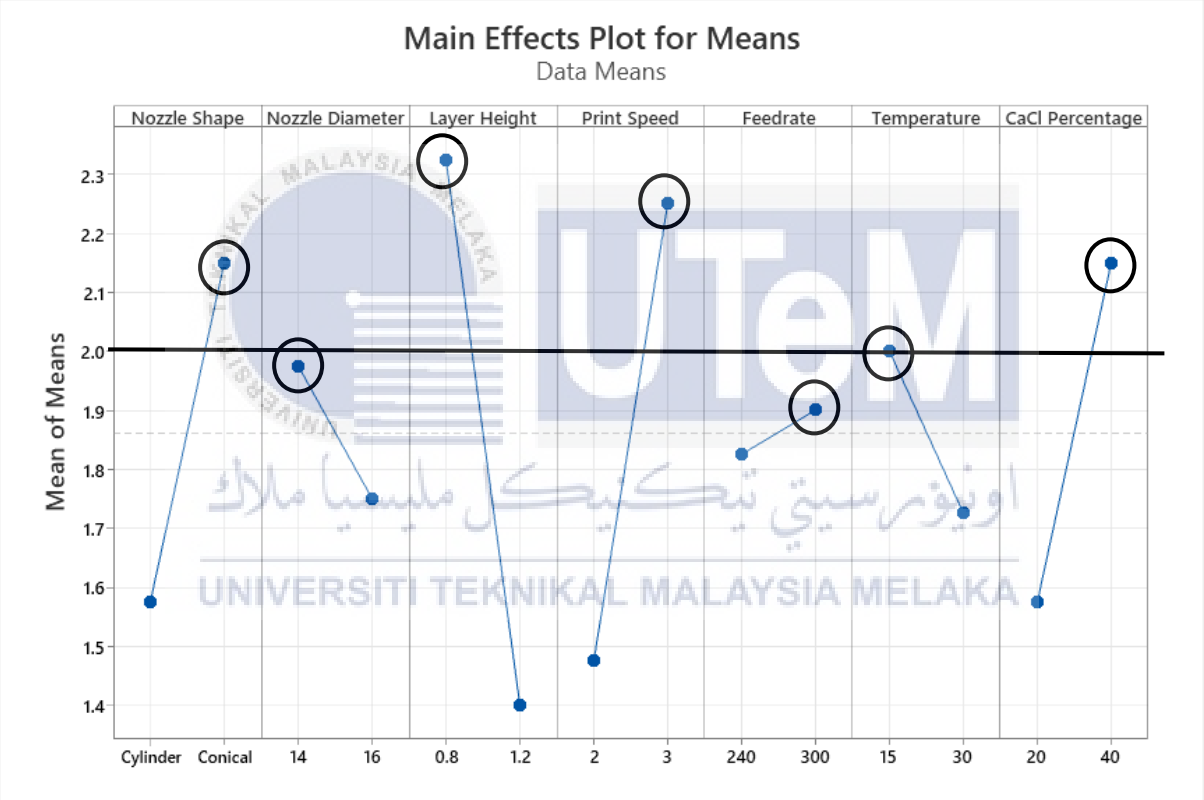


Figure 25: Response graph for mean value

The "Main Effects Plot for Means" graph in figure 25 generated using Minitab software by following the data from table 10 using nominal the best criteria. It provides insights into how various 3D printing parameters affect the mean Total Productive Maintenance (TPM). The graph indicates that the layer height and print speed have the most significant impacts on TPM due to longer line present, with smaller layer heights (0.8 mm) and higher print speeds (3 mm/s) considerably enhancing TPM where the point is near the nominal the best mean line which 2. The nozzle shape also plays a crucial role, with conical nozzles resulting in higher TPM than

cylindrical ones. While nozzle diameter and feed rate show some influence, their effects are relatively minor, with smaller diameters (14 mm) and higher feed rates (300 mm/min) only slightly improving TPM. Temperature and CaCl concentration have moderate impacts, with lower temperatures (15°C) and higher CaCl percentages (40%) contributing to better TPM. This analysis suggests that optimizing layer height, print speed, nozzle shape and CaCl concentration should be prioritized to improve TPM in 3D printing processes.

4.1.3 Validation of optimized printing parameters

Table 11: Optimized printing parameters

Nozzle Shape	Nozzle Size	Layer Height	Print Speed	Feed rate	Temperature	CaCl Percentage
Conical	14 ga	0.8 mm	3 mm/s	300 mm/s	15°C	40%

Table 11 show the optimized parameters for 3D printing hydrogels which are conical nozzle shape, 14-gauge nozzle size, 0.8 mm layer height, 3 mm/s print speed, 300 mm/min feed rate, 15°C temperature, and 40% CaCl concentration. The optimized parameter than used to print the sample as in figure 21 for validation process in result enhances continuous flow, structural integrity, and printing accuracy. The conical nozzle and small size ensure a consistent and smooth extrusion, reducing the risk of clogging and interruptions. A 0.8 mm layer height, combined with the higher print speed, balances deposition rate and precision, supporting a steady material flow. The 300 mm/min feed rate matches this flow, preventing gaps or over-extrusion. Structurally, the fine layer height and high CaCl concentration improve layer adhesion and cross-linking density, resulting in a robust and stable printed object. For accuracy, the conical nozzle and controlled print speed allow precise material deposition, while the lower temperature maintains optimal hydrogel viscosity. These parameters collectively ensure that the hydrogel objects are accurately printed, structurally sound, and produced with a smooth, continuous material flow.

Figure 26: Sample print with optimized parameter

4.2 Microstructure analysis using optical microscope

Comparing the microstructure of printed hydrogels in Figures 27 and 28 reveals noticeable differences in pore presence, represented as dark circles. Figure 27 shows a relatively smooth and compact surface using optical microscope with 50x magnification and scale bar of (80µm). Its texture is uniform with only a few irregularities. The visible pores are relatively few and larger in size, indicating a denser network structure. This limited porosity suggests that the hydrogel may have higher mechanical strength and reduced swelling capacity compared to more porous materials. The dense arrangement of polymer chains within the hydrogel contributes to its rigidity and potentially restricts the movement of water molecules within the matrix. Consequently, this hydrogel likely exhibits lower water absorption and slower swelling rates, making it suitable for applications where stability and minimal deformation are important.

In contrast, Figure 28 image shows a rougher surface texture with numerous small pores spread throughout. This highly porous structure suggests a more open and interconnected network, significantly increasing the surface area available for water or other substance interactions. The presence of many small pores indicates that this hydrogel is less dense and more flexible than Figure 27. The extensive porous network allows for rapid water absorption and swelling, making the hydrogel ideal for applications requiring high water content and quick response times. The increased porosity also implies lower mechanical strength but higher elasticity, allowing the hydrogel to deform easily under stress and return to its original shape once the stress is removed.

Both hydrogels with distinctly different microstructural characteristics, influencing their physical properties and potential applications. The first hydrogel, with its smoother surface and fewer, larger pores, is likely denser, less flexible, and exhibits slower swelling behaviour. This makes it suitable for applications where mechanical stability and reduced fluid uptake are important. In contrast, the second hydrogel's rougher surface and high density of small pores suggest a more porous and elastic material. This hydrogel is better suited for applications requiring high water absorption, rapid swelling, and flexibility, such as wound dressings, drug delivery systems, or soft tissue engineering scaffolds. Overall, both sets of hydrogel samples demonstrate pore presence, confirming porosity essential for cell growth, aligning with findings from other research in this area validate that the sample print with crosslinker module was successful.

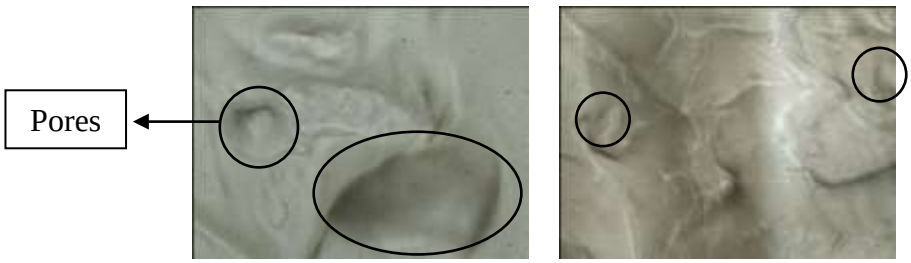


Figure 27: Microstructure of printed sample using optical microscope with 50x magnification

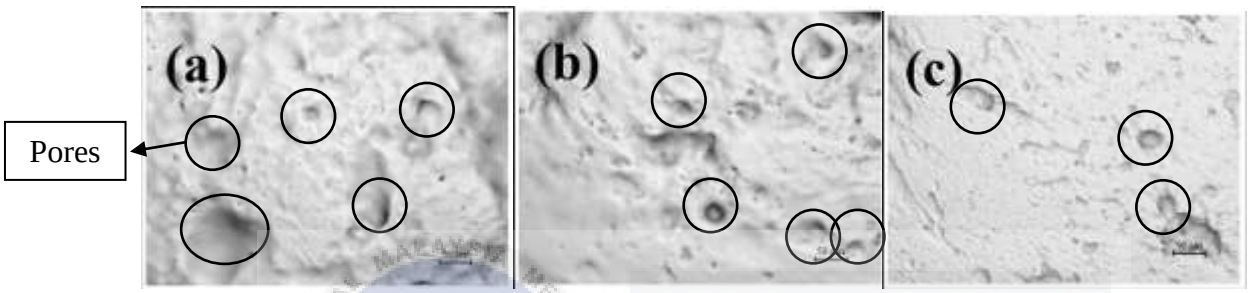


Figure 28: Optical Microscope Images of Hydrogels (A-c) (Kundu & Banerjee, 2020)

CHAPTER 5
CONCLUSION

In conclusion, the experimental objectives were successfully achieved, showcasing significant advancements in hydrogel bioprinting. The primary objective to devise a crosslinking module for hydrogel polymerization on the existing GelPrint Pro bioprinter was accomplished. This new crosslinking module was seamlessly integrated into the bioprinter, enhancing its capability to precisely control the polymerization process during different type of hydrogel printing.

The second objective, which focused on optimizing the bioprinting parameter with the crosslinker module, was also realized. Through systematic experimentation and refinement of printing parameters using orthogonal array $L_8(2^7)$, the optimized parameter ensured that the printed hydrogels maintained their structural integrity and microstructure. The optimized parameters facilitated a consistent and reliable bioprinting process, crucial for producing continuous flow of hydrogel printing, improve structure and high printing quality.

The final objective involved investigating the structural characteristics of the scaffolds built with the crosslinker module. Detailed analysis revealed that the new crosslinker module could print hydrogels without adversely affecting the microstructure of the scaffolds. The printed hydrogels exhibited desired porosity and uniformity, essential for their intended applications in tissue engineering and other biomedical fields.

Additionally, the study found that the double extrusion syringe system was superior to the support bath method. The double extrusion system proved to be easier to use and more efficient, allowing for better control over the printing process. This method's simplicity and effectiveness make it a preferred choice for bioprinting hydrogels, streamlining the workflow and reducing potential complications associated with more complex methods like the support bath.

Overall, this experiment not only met its objectives but also demonstrated that the new crosslinker module and the double extrusion syringe system together offer a robust and user-friendly solution for hydrogel bioprinting. This advancement paves the way for more precise and reliable fabrication of hydrogel scaffolds, which are crucial for various biomedical applications.

5.1 Limitation and Recommendation

Present notable challenges when used with double syringe extruder systems, especially for printing multiple layers and limited print direction due to limitation of hydrogel characteristic itself. CaCl is favoured for its ability to form stable crosslinks with hydrogel polymers, but it significantly alters the rheological properties of the hydrogel solution, increasing viscosity and affecting extrusion dynamics. This heightened viscosity can lead to difficulties in maintaining consistent extrusion rates and pressure, potentially causing uneven layer deposition or nozzle clogging during printing. Moreover, the polymerization initiated by CaCl can proceed rapidly, requiring precise control of extrusion parameters to ensure continuous printability throughout the layering process. Achieving strong interlayer adhesion poses another challenge, as CaCl crosslinked hydrogels may exhibit reduced bonding between layers, compromising the structural integrity of the printed object.

Addressing these challenges, it is recommended to use a coaxial nozzle as in Figure 29, in the context of printing hydrogels with a double syringe extruder system represents a sophisticated approach to enhancing the precision and functionality of bioprinted constructs. Unlike single-nozzle systems for each bioink and CaCl, coaxial nozzles facilitate simultaneous extrusion of multiple materials typically a hydrogel core surrounded by a sheath of crosslinking agent. It enables precise control over material deposition in any direction, which is critical for achieving intricate geometries and fine details in printed structures. By encapsulating the hydrogel within a supportive or crosslinking material during extrusion, coaxial nozzles help maintain the integrity of delicate features and minimize premature gelation. Overall, the use of coaxial nozzles in hydrogel bioprinting with a double syringe extruder system may represents a significant advancement in additive manufacturing technology, offering enhanced control, resolution, and functionality for creating complex tissue scaffolds and biomedical devices.

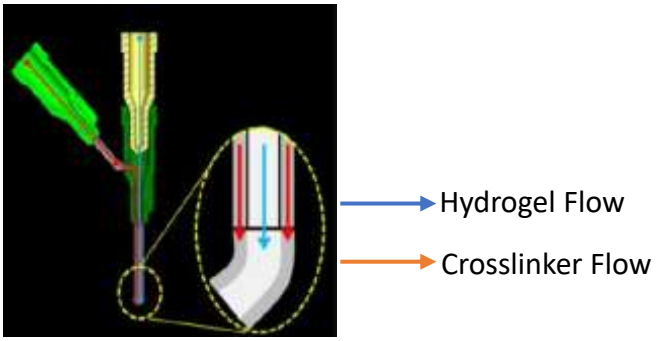


Figure 29: Coaxial nozzle working principle (Ye et al., 2022)

Using an orthogonal array to optimize printing parameters in hydrogel bioprinting also challenging, especially when evaluating results through scoring and visualization with the same sample across multiple experiments. Using the same biological sample introduces variability that might not be fully accounted for in analyses. Consistent and reproducible results are hard to achieve due to biological and experimental variability. Additionally, the scoring system based on visualization is unreliable because different people perceive visual details differently.

To improve the scoring technique for optimizing printing parameters in hydrogel bioprinting, several strategies can be used. Instead of using a single biological sample for multiple experiments, use multiple samples or appropriate controls to account for biological variability. This helps in getting a better assessment of how different parameter combinations affect printing outcomes. Additionally, a picture image method can be valuable. This involves taking high-resolution images of the printed structures with digital imaging technology. These images are then analysed both qualitatively and quantitatively. Qualitative assessment looks at surface smoothness, layer uniformity, shape fidelity, and defects like droplets or voids. Quantitative analysis uses software to measure parameters like dimensional accuracy, layer thickness, and pore size distribution. This objective analysis allows for precise comparisons between different printing conditions, helping to identify the best configurations for superior print outcomes.

Lastly, comparing optical microscopy and scanning electron microscopy (SEM) for characterizing hydrogel microstructure, SEMs offer higher resolving power and magnification, achieving sub-nanometre resolution and 100,000x magnification. Optical microscopy, on the other hand, is limited to around 200 nm resolution and 1,500x magnification. However, SEMs require drying the hydrogel using dry freezing which can alter the microstructure, while optical microscopy can image hydrogels in their native, hydrated state. SEMs produce grayscale images but can provide chemical composition information in more detail as clear sight of the pores such as shown in Figure 10, while optical microscopy produces colour images as show in Figure 27.

5.2 Sustainable Design and Development

The sustainable design and development of a hydrogel bioprinter employing a double syringe extruder system offers significant advantages over support-based solutions. The double syringe system enhances sustainability by minimizing material waste through precise deposition of hydrogels, reducing the need for additional sacrificial materials used in support-based solution methods. This efficiency not only conserves resources but also decreases landfill contributions, aligning with environmental goals. Moreover, the operational efficiency of the double syringe system contributes to lower energy consumption, where the preparation of support-based of calcium chloride used more energy due to many processes involved.

Utilizing PLA (Poly(lactic acid)) material in fabrication of double syringe extruder module amplifies these sustainability benefits. PLA is derived from renewable resources such as corn starch or sugarcane, reducing reliance on fossil fuels and lowering the carbon footprint associated with material production. Its biodegradability under industrial composting conditions provides a sustainable end-of-life option for bioprinted products, contrasting with non-biodegradable plastics that contribute to environmental pollution.

5.3 Complex Engineering Problem

Creating the crosslinker module in a hydrogel bioprinter is a complex engineering task, especially when comparing the double syringe extruder system to support-based solutions. The double syringe extruder system tackles these challenges by precisely controlling the deposition and integration of crosslinking agents into hydrogel materials. It ensures accurate mixing and distribution of crosslinkers like calcium ions, which are essential for maintaining the structure and bioactivity of printed tissues. This system is also adaptable to various hydrogel formulations and crosslinking agents, making it versatile for different tissue engineering applications. Its automated features improve scalability and efficiency, unlike support-based methods that require more manual work and can be less consistent. Overall, the double syringe extruder system represents a significant advancement in overcoming engineering challenges in bioprinting, enabling more reliable and reproducible creation of intricate tissue constructs for biomedical research and clinical use.

5.4 Commercialization Potential

The double syringe extruder system has strong commercial potential for making the crosslinker module in hydrogel bioprinters due to its precise control and versatility. It accurately places crosslinking agents in hydrogel materials, which is essential for creating consistent and functional tissue constructs. This precision is valuable for tissue engineering, drug delivery, and biomedical research. Its compatibility with various hydrogel formulations and crosslinking agents allows customization for specific biomedical needs, increasing its market appeal. The system's ability to automate and scale production reduces costs and boosts efficiency, making it attractive to research labs and biotech companies. Overall, the double syringe extruder system enhances precision, versatility, and scalability in bioprinting, making it a promising technology for advancing medical therapies and research applications.

REFERENCES

1. He, Y., Yang, F., Zhao, H., Gao, Q., Xia, B., & Fu, J. (2016). Research on the printability of hydrogels in 3D bioprinting. *Scientific Reports*, *6*(1). https://doi.org/10.1038/srep29977
2. Enrique Mancha Sánchez, J. Carlos Gómez-Blanco, Esther López Nieto, Casado, J. G., A. Macías-García, María A. Díaz Díez, Juan Pablo Carrasco-Amador, Diego Torrejón Martín, Sánchez-Margallo, F. M., & J. Blas Pagador. (2020). Hydrogels for Bioprinting: A Systematic Review of Hydrogels Synthesis, Bioprinting Parameters, and Bioprinted Structures Behavior. *Frontiers in Bioengineering and Biotechnology*, *8*. https://doi.org/10.3389/fbioe.2020.00776
3. Amin GhavamiNejad, Nureddin Ashammakhi, Xiao Yu Wu, & Khademhosseini, A. (2020). Crosslinking Strategies for 3D Bioprinting of Polymeric Hydrogels. *Small*, *16*(35). https://doi.org/10.1002/smll.202002931
4. Nelson, C., Slesha Tuladhar, Launen, L. A., & Habib, A. (2021). 3D Bio-Printability of Hybrid Pre-Crosslinked Hydrogels. *International Journal of Molecular Sciences*, *22*(24), 13481–13481. https://doi.org/10.3390/ijms222413481
5. Bialik-Wąs, K., Królicka, E., & Malina, D. (2021). Impact of the Type of Crosslinking Agents on the Properties of Modified Sodium Alginate/Poly(vinyl Alcohol) Hydrogels. *Molecules*, *26*(8), 2381. https://doi.org/10.3390/molecules26082381
6. Saman Naghieh, & Chen, D. (2021). Printability–A key issue in extrusion-based bioprinting. *Journal of Pharmaceutical Analysis*, *11*(5), 564–579. https://doi.org/10.1016/j.jpha.2021.02.001
7. Al-Sabah, A., Stephanie E.A. Burnell, Simões, I. N., Jessop, Z. M., Nafiseh Badiei, Emma Jane Blain, & Whitaker, I. S. (2019). Structural and mechanical characterization of crosslinked and sterilised nanocellulose-based hydrogels for cartilage tissue engineering. *Carbohydrate Polymers*, *212*, 242–251. https://doi.org/10.1016/j.carbpol.2019.02.057

8. Knowlton, S., Bekir Yenilmez, Anand, S., & Savaş Tasoğlu. (2017). Photocrosslinking-based bioprinting: Examining crosslinking schemes. *Bioprinting*, *5*, 10–18. https://doi.org/10.1016/j.bprint.2017.03.001
9. Zennifer, A., Sweda Manivannan, Sethuraman, S., Kumbar, S. G., & Dhakshinamoorthy Sundaramurthi. (2022). 3D bioprinting and photocrosslinking: emerging strategies & future perspectives. *Biomaterials Advances*, *134*, 112576–112576. https://doi.org/10.1016/j.msec.2021.112576
10. Sriya Yeleswarapu, Dash, A., Shibu Chameettachal, & Pati, F. (2023). 3D bioprinting of tissue constructs employing dual crosslinking of decellularized extracellular matrix hydrogel. *Biomaterials Advances*, *152*, 213494–213494. https://doi.org/10.1016/j.bioadv.2023.213494
11. Mabel Barreiro Carpio, Mohammadhossein Dabaghi, Ungureanu, J., & Moran-Mirabal, J. M. (2021, November 24). *3D Bioprinting Strategies, Challenges, and Opportunities to Model the Lung Tissue Microenvironment and Its...* ResearchGate; Frontiers. https://www.researchgate.net/publication/356651304_3D_Bioprinting_Strategies_Challenges_and_Opportunities_to_Model_the_Lung_Tissue_Microenvironment_and_Its_Function
12. Saini, G., Segaran, N., Mayer, J. L., Saini, A., Albadawi, H., & Rahmi Öklü. (2021). Applications of 3D Bioprinting in Tissue Engineering and Regenerative Medicine. *Journal of Clinical Medicine*, *10*(21), 4966–4966. https://doi.org/10.3390/jcm10214966
13. Agarwal, S., Saha, S., Vamsi Krishna Balla, Pal, A., Ananya Barui, & Subhadip Bodhak. (2020). Current Developments in 3D Bioprinting for Tissue and Organ Regeneration–A Review. *Frontiers in Mechanical Engineering*, *6*. https://doi.org/10.3389/fmech.2020.589171
14. Wu, C. A., Zhu, Y., & Y. Joseph Woo. (2023). Advances in 3D Bioprinting: Techniques, Applications, and Future Directions for Cardiac Tissue Engineering. *Bioengineering*, *10*(7), 842–842. https://doi.org/10.3390/bioengineering10070842

15. Kotlarz, M., Ana Marina Ferreira, Gentile, P., Russell, S. J., & Dalgarno, K. (2022). Droplet-based bioprinting enables the fabrication of cell–hydrogel–microfibre composite tissue precursors. *Bio-Design and Manufacturing*, *5*(3), 512–528. https://doi.org/10.1007/s42242-022-00192-5
16. Saeedeh Vanaei, Mohammad Salemizadeh Parizi, Fatemeh Salemizadehparizi, & Hamid Reza Vanaei. (2021). An Overview on Materials and Techniques in 3D Bioprinting Toward Biomedical Application. *Engineered Regeneration*, *2*, 1–18. https://doi.org/10.1016/j.engreg.2020.12.001
17. Li, W., Wang, M., Ma, H., Chapa-Villarreal, F. A., Anderson Oliveira Lobo, & Yu Shrike Zhang. (2023). Stereolithography apparatus and digital light processing-based 3D bioprinting for tissue fabrication. *IScience*, *26*(2), 106039–106039. https://doi.org/10.1016/j.isci.2023.106039
18. Rider, P., Željka Perić Kačarević, Said Alkildani, Sujith Retnasingh, & Barbeck, M. (2018). Bioprinting of tissue engineering scaffolds. *Journal of Tissue Engineering*, *9*, 204173141880209-204173141880209. https://doi.org/10.1177/2041731418802090
19. Papaioannou, T. G., Manolesou, D., Evangelos Dimakakos, Tsoucalas, G., Manolis Vavuranakis, & Dimitrios Tousoulis. (2019). 3D Bioprinting Methods and Techniques:Applications on Artificial Blood Vessel Fabrication. *PubMed*, *35*(3), 284–289. https://doi.org/10.6515/acs.201905_35(3).20181115a
20. Mohamadmahdi Samandari, Farnoosh Saeedinejad, Quint, J., Xin, S., Farzad, R., & Tamayol, A. (2023). Repurposing biomedical muscle tissue engineering for cellular agriculture: challenges and opportunities. *Trends in Biotechnology*, *41*(7), 887–906. https://doi.org/10.1016/j.tibtech.2023.02.002
21. Sachdev, A., Acharya, S., Tejas Gadodia, Shukla, S., J Harshita, Akre, C., Khare, M., & Huse, S. (2022). A Review on Techniques and Biomaterials Used in 3D Bioprinting. *Cureus*. https://doi.org/10.7759/cureus.28463
22. Dell, A. C., Wagner, G. A., Own, J., & Geibel, J. P. (2022). 3D Bioprinting Using Hydrogels: Cell Inks and Tissue Engineering Applications. *Pharmaceutics*, *14*(12), 2596–2596. https://doi.org/10.3390/pharmaceutics14122596

23. G. González-Ulloa, Jiménez-Rosado, M., Mohammed Rafii-El-Idrissi Benhnia, Romero, A., Ruiz-Mateos, E., Francisco José Ostos, & Víctor Pérez-Puyana. (2023). Hybrid polymeric Hydrogel-based biomaterials with potential applications in regenerative medicine. *Journal of Molecular Liquids*, *384*, 122224–122224. https://doi.org/10.1016/j.molliq.2023.122224
24. Ding, Y., Zhang, X.-W., Mi, C.-H., Qi, X.-Y., Zhou, J., & Wei, D. (2023). Recent advances in hyaluronic acid-based hydrogels for 3D bioprinting in tissue engineering applications. *Smart Materials in Medicine*, *4*, 59–68. https://doi.org/10.1016/j.smaim.2022.07.003
25. Gu, Z., Fu, J., Lin, H., & He, Y. (2020). Development of 3D bioprinting: From printing methods to biomedical applications. *Asian Journal of Pharmaceutical Sciences*, *15*(5), 529–557. https://doi.org/10.1016/j.ajps.2019.11.003
26. Xu, Y., Wang, C., Yang, Y., Liu, H., Xiong, Z., Zhang, T., & Sun, W. (2022). A Multifunctional 3D Bioprinting System for Construction of Complex Tissue Structure Scaffolds: Design and Application. *International Journal of Bioprinting*, *8*(4). https://doi.org/10.18063/ijb.v8i4.617
27. Deo, K. A., Kanwar Abhay Singh, Peak, C. W., Alge, D. L., & Gaharwar, A. K. (2020). Bioprinting 101: Design, Fabrication, and Evaluation of Cell-Laden 3D Bioprinted Scaffolds. *Tissue Engineering Part A*, *26*(5-6), 318–338. https://doi.org/10.1089/ten.tea.2019.0298
28. M. Azad Emin, Wittek, P., & Schwegler, Y. (2021). Numerical analysis of thermal and mechanical stress profile during the extrusion processing of plasticized starch by non-isothermal flow simulation. *Journal of Food Engineering*, *294*, 110407–110407. https://doi.org/10.1016/j.jfoodeng.2020.110407
29. Iliyana Pepelanova, Kruppa, K., Scheper, T., & Lavrentieva, A. (2018). Gelatin-Methacryloyl (GelMA) Hydrogels with Defined Degree of Functionalization as a Versatile Toolkit for 3D Cell Culture and Extrusion Bioprinting. *Bioengineering*, *5*(3), 55–55. https://doi.org/10.3390/bioengineering5030055

30. Wei, F., Yang, M., Wang, L., Li, W., Liu, M., Jin, Y., Wang, Y., Yang, R., Wang, Y., Zhang, K., & Fu, Q. (2023). Hydrogels for 3D bioprinting in tissue engineering and regenerative medicine: Current progress and challenges. *International Journal of Bioprinting*, *9*(5), 759–759. https://doi.org/10.18063/ijb.759
31. Sang Cheon Lee, Gillispie, G. J., Prim, P. M., & Sang Jin Lee. (2020). Physical and Chemical Factors Influencing the Printability of Hydrogel-based Extrusion Bioinks. *Chemical Reviews*, *120*(19), 10834–10886. https://doi.org/10.1021/acs.chemrev.0c00015
32. Webb, B., & Doyle, B. (2017). Parameter optimization for 3D bioprinting of hydrogels. *Bioprinting*, *8*, 8–12. https://doi.org/10.1016/j.bprint.2017.09.001
33. Leberfinger, A. N., Dinda, S., Wu, Y., Srinivas Koduru, Veli Özbolat, Ravnic, D. J., & Özbolat, İ. T. (2019). Bioprinting functional tissues. *Acta Biomaterialia*, *95*, 32–49. https://doi.org/10.1016/j.actbio.2019.01.009
34. He, Y., Gu, Z., Xie, M., Fu, J., & Lin, H. (2020). Why choose 3D bioprinting? Part II: methods and bioprinters. *Bio-Design and Manufacturing*, *3*(1), 1–4. https://doi.org/10.1007/s42242-020-00064-w
35. Koch, M., & Włodarczyk-Biegun, M. K. (2020). Faithful scanning electron microscopic (SEM) visualization of 3D printed alginate-based scaffolds. *Bioprinting*, *20*, e00098–e00098. https://doi.org/10.1016/j.bprint.2020.e00098
36. Kaczmarek, B., Miłek, O., Michalska-Sionkowska, M., Zasada, L., Twardowska, M., Warżyńska, O., Kleszczyński, K., & Osyczka, A. M. (2020). Novel eco-friendly tannic acid-enriched hydrogels-preparation and characterization for biomedical application. Materials, 13(20), 1–12. https://doi.org/10.3390/ma13204572
37. Nafar Dastgerdi, J., Koivisto, J. T., Orell, O., Rava, P., Jokinen, J., Kanerva, M., & Kellomäki, M. (2021). Comprehensive characterisation of the compressive behaviour of hydrogels using a new modelling procedure and redefining compression testing. Materials Today Communications, 28. https://doi.org/10.1016/j.mtcomm.2021.102518

38. Magdalena Beata Łabowska, Skrodzka, M., Sicińska, H., Michalak, I., & Jerzy Detyna. (2023). Influence of Cross-Linking Conditions on Drying Kinetics of Alginate Hydrogel. *Gels*, *9*(1), 63–63. https://doi.org/10.3390/gels9010063
39. Rasheed, A., Azizi, L., Turkki, P., Janka, M., Hytönen, V. P., & Sampo Tuukkanen. (2020). Extrusion-Based Bioprinting of Multilayered Nanocellulose Constructs for Cell Cultivation Using*In Situ*Freezing and Preprint $CaCl_2$Cross-Linking. *ACS Omega*, *6*(1), 569–578. https://doi.org/10.1021/acsomega.0c05036
40. Zerihun Feyissa, Gemechu Deressa Edossa, Gupta, N., & Defaru Negera. (2023). Development of double crosslinked sodium alginate/chitosan based hydrogels for controlled release of metronidazole and its antibacterial activity. *Heliyon*, *9*(9), e20144–e20144. https://doi.org/10.1016/j.heliyon.2023.e20144
41. Ann Abert, A., Akiah, M.-A., Kejuruteraan Pembuatan, F., Teknikal Malaysia Melaka, U., Tuah Jaya, H., & Tunggal, D. (2022). Development of modular-based bioprinter with three-way valve for hydrogel control.
42. Ye, M., Lu, B., Zhang, X., Li, B., Xiong, Z., & Zhang, T. (2022). Coaxial Embedded Printing of Gelatin Methacryloyl–alginate Double Network Hydrogel for Multilayer Vascular Tubes. *Chinese Journal of Mechanical Engineering. Additive Manufacturing Frontiers*, *1*(2), 100024–100024. https://doi.org/10.1016/j.cjmeam.2022.100024
43. Kundu, D., & Banerjee, T. (2020). Development of microcrystalline cellulose based hydrogels for the in vitro delivery of Cephalexin. Heliyon, 6(1). https://doi.org/10.1016/j.heliyon.2019.e03027
44. KENNEDY MEKANTUS (2023). APPLICATION OF SEAWEED-BASED HYDROGEL FOR BIO-PRINTING KENNEDY MEKANTUS B051920133 UNIVERSITI TEKNIKAL MALAYSIA MELAKA 2023.
45. Masni Azian, & Abernice Anne. (2023). INNOVATION PITCH COMPETITION CHESS SYMPOSIUM 2023 Empowering Youth, Unlocking the Future.