

DEVELOPMENT OF A 3D BIOPRINTING SYSTEM, CHARACTERISATION
OF BIOINK AND PRINTING MICROSPHEROIDS OF HELA CELLS

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To those who have always been there for me;
my parents, my siblings, uncle and grandfather.



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ABSTRACT

3D Bioprinting is a promising technology widely applicable to the biomedical engineering field. This technology provides an advance alternative in terms of tissue and organ fabrication that will soon surpass the traditional 2D cell culture methods. Various of existing 3D bioprinting techniques had been developed such as extrusion-based, inkjet-based, laser assisted deposition and stereolithography. The existing microextrusion-based technology is designed with complex system and printing of scattered cells which presented threats to the cell viability. To circumvent this problem, a microextrusion-based 3D bioprinting system that implements the printing of microtissues was developed. In this project, the mechatronic system of the extruder was fabricated and programmed with flow rates of 1, 2, 3, 4 and 5 ml/min. Then, it was assembled to the existing 3D printer to function as a 3D bioprinter. The bioprinter moves at a printing speed set between 10 to 50 mm/s based on the G-codes designed for the desired structures. The effects of flow rate of the extruder and printing speed of the bioprinter to the printability of bioink was determined by examining the linearity in width of the printed constructs captured in images. The viscosities of five different concentrations of sodium alginate-gelatin bioink were determined by vibration viscometer. The bioprinting inks were also characterised using contact angle, Fourier Transform Infrared Spectroscopy (FTIR) and Dynamic Mechanical Analysis (DMA). The degradation of bioink was observed for 14 days. After the printing parameters were optimised, constructs of alginate-gelatin were printed with HeLa cells infused in calcium alginate microcapsules. After 14 days of culture, the tissues were still viable as indicated by the green fluorescence of calcein-acetoxymethyl. The printed biofilament are potential cell models that can be utilised for the study of oncology and pharmacology. The bioprinting system was successfully developed and applied to print the encapsulated 3D microtissues into 3D constructs.

ABSTRAK

Pencetak bio 3D adalah satu teknologi harapan yang digunapakai secara meluas dalam bidang kejuruteraan bioperubatan. Teknologi ini menyediakan satu alternatif yang maju dari segi fabrikasi tisu dan organ yang bakal melepasi kaedah tradisional dua-dimensi (2D). Pelbagai teknik pencetak bio 3D yang sedia ada telah dibangunkan seperti pencetak berasaskan pengestrusi mikro, pancut dakwat, pemendapan laser dan stereolitografi. Kaedah pengestrusi mikro sedia ada telah direka dengan sistem yang kompleks dan pencetakan sel secara berselerak telah memberikan ancaman kepada jangka hayat sel. Untuk mengelakkan masalah ini, satu sistem pencetak bio 3D berasaskan kaedah pengestrusi mikro yang mampu melaksanakan percetakan tisu mikro telah dibangunkan. Dalam projek ini, sistem mekatronik pengestrusi direka dan diprogramkan dengan kadar aliran 1, 2, 3, 4 dan 5 ml/min. Kemudian, ia telah dipasangkan pada pencetak 3D sedia ada untuk berfungsi sebagai pencetak bio 3D. Pencetak bio tersebut bergerak mengikut kelajuan percetakan yang ditetapkan antara 10 hingga 50 mm/s berdasarkan kod G yang direka untuk struktur yang dikehendaki. Kesan kadar aliran dan kelajuan percetakan pencetak bio kepada kebolehcetakan dakwat bio ditentukan dengan memeriksa kerataan dalam lebar binaan yang ditangkap dalam imej. Kelikatan lima kepekatan dakwat bio alginat-gelatin diukur dengan menggunakan viskometer getaran. Dakwat pencetak bio juga dicirikan menggunakan sudut sentuhan, spektroskopi inframerah transformasi Fourier (FTIR), dan analisis mekanikal dinamik (DMA). Degradasi dakwat bio telah diperhatikan selama 14 hari. Setelah kesemua parameter percetakan dioptimumkan, binaan natrium alginat/gelatin telah dicetak bersama sel *HeLa* yang telah dicampurkan dalam mikrokapsul kalsium alginat. Selepas pengulturan selama 14 hari, tisu tersebut didapati masih berdaya maju seperti yang ditunjukkan oleh fluoresens hijau *calcein-acetoxymethyl*. Filamen bio yang telah dicetak adalah model sel berpotensi yang boleh diadaptasikan dalam kajian onkologi dan farmakologi. Sistem percetakan bio telah berjaya dibangunkan dan digunakan untuk mencetak kapsul mikrotisu 3D kepada binaan 3D

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LIST OF SYMBOLS AND ABBREVIATIONS

μm	-	Micrometer
2D	-	Two-dimensional
3D	-	Three-dimensional
ABS	-	Acrylonitrile Butadiene Styrene
CaCl	-	Calcium Chloride
CAD	-	Computer-Aided Design
Calcein AM	-	Calcein Acetoxymethyl
CHO	-	Chinese Hamster Ovary
cm	-	Centimeter
CNC	-	Computer Numerical Control
DIR	-	Direction pin
DMA	-	Dynamic Mechanical Analysis
DMEM	-	Dulbecco's Modified Eagle's Medium
ECM	-	Extracellular Matrix
EDTA Trypsin	-	Ethylenediaminetetraacetic acid Trypsin
ENA	-	Enable pin
ER	-	Endoplasmic Reticulum
EthD-1	-	Ethidium homodimer-1
FDM	-	Fused Deposition Modelling
FTIR	-	Fourier Transform Infrared Spectroscopy
g	-	Gram
G'	-	Storage modulus
G''	-	Loss modulus
Gel	-	Gelatin
GelMa	-	Gelatin Methacrylate
HA	-	Hyaluronic Acid
HBSS	-	Hank's Balance Salt Solution
HeLa	-	Henrietta Lacks cervical cell line

HPV	-	Human Papillomavirus
Hz	-	Hertz
K	-	Spring constant
kDa	-	KiloDalton
LAB	-	Laser-Assisted Bioprinting
LCD	-	Liquid Crystal Display
LED	-	Light-Emitting Diode
m	-	Mass
mg	-	Milligram
min	-	Minute
ml	-	Milliliter
mm	-	Millimeter
MPa	-	Megapascal
NaAlg	-	Sodium Alginate
Pa	-	Pascal
PEG	-	Polyethylene Glycol
PEGDA	-	Poly(ethylene glycol) Diacrylate
PEGDMA	-	Poly (ethylene glycol) Dimethacrylate
PLA	-	Poly(lactic) Acid
PLGA	-	Poly(lactic-co-glycolic) Acid
PUL	-	Pulse pin
RPM	-	Rotation Per Minute
s	-	Second
STL	-	Stereolithography
USB	-	Universal Serial Bus
UV	-	Ultraviolet
v/v	-	Volume per volume
W	-	Weight
w/v	-	Weight per volume
x	-	Amplitude
θ	-	Theta
ω_n	-	Natural frequency

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CHAPTER 1

INTRODUCTION

1.1 Overview

The need of organ and insufficient supply of organ trigger the need of regenerating artificial organ or tissues. Various biotechnologies are undertaken by scientist and engineers to develop breakthrough products for minimising health risks and life-threatening conditions. These technologies are such as bioinformatics, biopharmaceutical, chemical industrial technology, organ regeneration and tissue engineering. Engineering solutions such as bioprinting in organ regeneration technology enable the hope to regenerate functional tissues for building more complex structures such as a pumping heart. 3D bioprinting involving the biomaterials such as synthetic and non-synthetic bioink and cells are critical factors in creating a better cell model study. In conventional cell culture routine, the two-dimensional (2D) cancer model is commonly used by the cell biologists and pharmacologist in research. However, currently, it is gaining controversy regarding the accuracy of the model for disease and oncology studies.

1.2 Research background

Till date, conventional two-dimensional (2D) cell models are still being used in biological studies to understand the cell formation, functions and pathology [1]. Although growing a flat layer of cells in 2D culture had contributed a lot in cell research, but it lacks of predictivity and accuracy which increases the cost and failure rate during clinical trials [2]. These monolayer cell cultures show limitations in terms of cell proliferation, differentiation, gene expression, cancer cell invasion and morphology which slightly differ from the *in vivo* state [3]. In contrary, the three-

dimensional (3D) cells are able to adequately produce similar cell behaviour and mimic closely to the microenvironment of the *in vivo* responses [4]. The 3D cell model has proven to be more physiologically relevant in proliferation, cell number monitoring, viability and other studies of biological mechanism [3]. This native-like 3D cells are becoming crucial scientific tools in cell research. Hence, researchers are encouraged to develop new techniques equipped with the capacity to produce 3D cell models such as 3D bioprinting technology.

3D bioprinting involves the three-dimensional printing of biological tissues and organs through the process of layering the living cells which begins by creating an architectural design of the fundamental composition of the target tissue or organ with the help of computer-aided design (CAD) [5]. The 3D printer that applies in the bioprinting is commonly known as 3D bioprinter. The bioprinter will deposit thin layer of cells based on the architectural design using a printer head which moves according to the required configuration. The bioprinter uses an ink called as the bioink to build the three-dimensional structure of the organic materials. The bioink can be synthetic and non-synthetic biomaterials mostly made up of dissolvable hydrogel component such as collagen, chitosan, Pluronic and hyaluronic acid (HA) [6]. Hydrogel is a hydrophilic network of polymer chains which is highly absorbent and possess a flexibility that is similar to the natural tissues [7]. This hydrogel is considered to have a great potential in medical purposes due to its ability to absorb large quantity of water, thus, in bioprinting, it is often used to support and protect the cells as well as acting as fillers to fill empty spaces within the tissues [8].

The application of bioprinting technology in biomedical field was inspired from the early development of three-dimensional printing system back in the 1900s [9]. It has been rapidly commercialised due to the significant interests gained from biomaterials development, medicine and pharmaceuticals industries. The current market offers bioprinters such as Organovo ExVive™, Aether I, FABION and BioBot™ Basic which work similarly but differs by the printing mechanism and bioink used [10], [11]. This rising technology had been adopted in tissue engineering and regenerative medicine into creating functional living tissues and organs. It has a great potential in keeping the pace with the growing demands for suitable organs and acts as an alternative to organ transplantation [12]. This new approach is able to create a structural and molecular environment that mimics significantly the properties of the native organ in order to support the recipient's cells. The 3D bioprinting allows the

accurate evaluation of cell microenvironment that accelerates the development of cancer therapeutics in oncology studies [13]. The 3D tissue model produced by bioprinting techniques can also be used in medicine and pharmacology research for drug treatments and study diseases [14].

There are four types of bioprinting that had been developed to fabricate the 3D cells which are stereolithography bioprinting [15][16], drop-on-demand or inkjet bioprinting [17][18], extrusion bioprinting [19][20] and laser-assisted bioprinting [21][22]. Stereolithography bioprinting works with photopolymers which are not stable over time and might harm the cell. For inkjet-based bioprinting, the droplets tend to spread inconsistently and unequal in size [23]. Previous research on laser-assisted bioprinting showed that this method did not suffer any nozzle blockages due to the nozzle-less printing setup [24]. However, it is an expensive method to use.

Amongst previous bioprinting techniques mentioned, the extrusion-based bioprinting is the simplest and effective ways to print wide range of biomaterials viscosity. It also promotes a short fabrication time than the other bioprinting techniques. From previous work, extrusion-based bioprinter is mostly applied for printing scattered single cells laden in the bioink which can be difficult for cell to communicate due to the long distance between each other [25].

Thus, this research proposed the inclusion of microtissues that simulate the tumour embedded deep in the tissues of approximately 1 mm thick. This project focuses on developing an extrusion-based bioprinter with a potential to extrude and print 3D microtissues capsules coated with sodium alginate and gelatin 3D hydrogels or the bioink. The bioinks were synthesised and characterised for the physical and mechanical properties. The microspheroids of HeLa or cervical cancer cells had been encapsulated first in calcium alginate using a flicking machine that could produce spheroids of microtissues. These spheroids were doped in the sodium alginate and gelatin bioink and printed into the 3D constructs. Calcium chloride (CaCl_2) had been used as the crosslinker of the printed hydrogel structures. Hence, this work is able to be applied for more precise prediction of drugs diffusion through multilayer structures of the tissues in pharmacological studies.

1.3 Problem statement

The two-dimensional (2D) cancer cell has been used for a long time as the physiological cancer model. In previous research [26], the 2D cell model had led to a nonpredictive data for the in vivo responses as it provided a poor development of model. Due to that, three-dimensional (3D) cancer cell model had been introduced in clinical trials [27]. The 3D model cells are known to be more novel and has the ability to represent the integral missing component in the in vitro cancer research. It is a good approach to grow and treat cancer tumour as it exhibits similar growth and treatment patterns [28]. It also helps to reduce the usage of animal models. Previous extrusion-based bioprinting technology had been developed to print 3D constructs with scattered cells technique [29]. The scattering of cells had contributed to the decrease of cell interaction because of the distance between each other in the 3D constructs. Besides that, most of the extrusion-based bioprinting used small diameter of nozzle in the range between 0.2 mm to 0.8 mm which produced good resolution or structure of printed filament. However, this will increase the shear stress on the nozzle tip which leads to low cell viability [30].

Therefore, this project is proposed by developing an extrusion-based 3D bioprinting system that capable to extrude 3D structures made up from sodium alginate and gelatin mixture (sodium alginate-gelatin). The mixture was to enhance the printability properties of the bioink to print stable 3D constructs. A 1 mm diameter of nozzle was used to reduce the shear stress on the tip during extrusion and able to improve the cell viability. The straight-forward controller is easy to use and operated well. Instead of using the scattered cells technique, a different approach was used to print structure that consists of microbeads of calcium alginate for the microencapsulation of HeLa cancer cells. This approach is to fuse the functional microtissues in sodium alginate - gelatin bioink 3D structure which capable to induce the cell interaction. The biomaterials from the bioink are also non-toxic and highly biocompatible natural polymers which will increase the viability of the cells [31].

1.4 Objectives of the research

The main objectives of the research are:

- i. To design and fabricate extrusion system to the existing 3D printer to form a 3D bioprinter.
- ii. To characterise the bioink properties and optimum printing speed with flow rate of the sodium alginate - gelatin bioink for a printable single and multi-layered filament.
- iii. To analyse the viability of the microencapsulated HeLa cells in the printed sodium alginate-gelatin biofilament.

1.5 Scopes

In order to achieve the stated objectives, the scopes of this project are planned as following:

- i. The design of the extrusion system with an extruder, controller and syringe to load the bioink by using computer aided design (CAD) software, Google SketchUp 2017.
- ii. The development of the system controller with selection buttons to control the flow rate of bioink.
- iii. The integration of the extrusion system with existing Anycubic Prusa i3 3D printer by using silicon tube of 300 mm long (Diameter = 2.5 mm) attached with nozzle tip (Diameter = 1 mm).
- iv. The calibration of the bioink flow rates and the printing speed of 3D bioprinter to produce desired line width.
- v. The synthesis of bioink with sodium alginate and gelatin composites.
- vi. The characterisation of bioink by using viscometer, contact angle, dynamic mechanical analysis device (DMA), Fourier transform infrared spectroscopy (FTIR) and degradation test.
- vii. The printing of layers of tubular structure of the bioink with encapsulation of microtissue inside the bioink.
- viii. The live and dead observation of the printed construct with microtissues.

1.6 Thesis contributions

In this dissertation, the experimental finding of a novel extrusion 3D bioprinting system and its application revealed a different approach in the tissue engineering field. The contributions of this study are listed below:

(a) An extrusion-based 3D bioprinting method

The bioprinter was built by integration of a controller, extruder and a commercial 3D printer. The tip holder and the syringe holder were also designed by CAD software and built by using the 3D printer. This bioprinting method is able to operate easily and user-friendly. The biocompatible bioink was successfully synthesized with suitable chemical, physical and mechanical properties and characterised by FTIR analysis, degradation test, contact angle measurement, viscosity and DMA analysis.

(b) Reduce animal model exploitation in cancer research and pharmaceuticals

The findings obtained have contributed to the understanding of the effectiveness of alginate/gelatin biopolymer to be use as bioink. The printability of multilayer structures in this experiment proved the sturdiness of the sodium alginate/gelatin bioink. The multilayer structures can also help in cancer research and pharmaceutical study such as drug treatment. This will contribute to the decreasing in exploitation of animal model and the cost in maintaining the animal houses.

(c) Printing 3D model with microencapsulated tissues

Build model bridging 2D and animal models by printing 3D filament laden with microencapsulated tissues which bring one step closer to the in-vivo model. Instead of the conventional scattered cells, printing 3D model with inclusion of microencapsulated tissues contributed to the increasing of cell to cell interaction and cell to matrix interaction.

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