DEVELOPMENT OF AN ELECTRONIC AEROSOL ATOMISATION SYSTEM FOR
GENERATING THREE-DIMENSIONAL (3D) CELLS IN
MICROENCAPSULATIONS AND MICROTISSUES CHARACTERISATION

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fulfilment of the requirement for the award of the
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Faculty of Electrical and Electronic Engineering
Universiti Tun Hussien Onn Malaysia

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Special dedication with full gratitude on the guidance and encouragement to families who loved, especially my beloved father and mother and not forgotten to my supervisor that contributed ideas and opinions
ACKNOWLEDGEMENT

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My special appreciations to my parents, family members and friends who has encourage, support, love, patience and understanding me throughout my involvement in this research project. Thank you for all the encouragement and affection given.

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We acknowledge Professor Cheong Sok Cheng from Cancer Research Malaysia for her kind contribution of oral squamous cell carcinoma (OSCC) cell line (ORL-48).
LIST OF ASSOCIATED PUBLICATIONS

Journal


ABSTRACT

Cell encapsulation is a micro technology widely applied in cell and tissue engineering, tissue transplantation and regenerative medicine. Various techniques had been developed for microencapsulation of cells but these techniques presented threat to the cells due to the harsh or chemical treatment applied. In this research, a simple and economic electronic aerosol atomisation system was proposed for producing calcium alginate microcapsules. The system was developed with the incorporation of a conventional syringe pump, a customised air pump and motor controller circuits. The microcapsules and 3D microtissues were biophysically characterised. For the output of the system, the microcapsules size slightly increased with the extrusion rates and decreased significantly with the airflow rates. At an extrusion rate of 20 µl/min and airflow rate of 0.3 l/min, microcapsules with a diameter ranging from 220 - 270 µm were generated. The polymerisation time for the microcapsules was approximately 10 minutes after the immersion in calcium chloride solutions. The microcapsules showed high porous surface structure in field emission-scanning electron microscopy (FE-SEM) imaging. Keratinocytes (HaCaT) and Oral Squamous Cell Carcinoma (ORL-48) cells at cell densities of $3 \times 10^7$ and $9 \times 10^7$ cells/ml, respectively were applied for encapsulation and successfully grew into microtissues after 16 days of culture. The fourier transform infrared (FTIR) spectroscopy of the 3D cells showed stretching in phosphate bond of Deoxyribonucleic acid (DNA) and Ribonucleic acid (RNA) backbone, lipid and protein. The cells of HaCaT and ORL-48 microtissues were viable and they were characterised by different nucleus size. Replating experiment demonstrated that the cells in the microtissues could spread and proliferate in the culture dish. The electronic aerosol atomisation system developed in this work has successfully produced microcapsules with controllable size and applicable for growing microtissues. The microtissues produced are potentially a useful cell model for the study of cytochemicals.
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<tbody>
<tr>
<td>2D</td>
<td>Two-Dimensional</td>
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<td>3D</td>
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<td>α</td>
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<tr>
<td>°C</td>
<td>Degree Celsius</td>
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<td>&lt;</td>
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<td>cells/ml</td>
<td>Cells per Milli Litre</td>
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<tr>
<td>cm</td>
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<td>cm²</td>
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<td>f</td>
<td>Frequency</td>
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<tr>
<td>cm⁻¹</td>
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<td>kg/m³</td>
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<td>CaCl$_2$</td>
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<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
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<td>Extracellular Matrix</td>
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<td>FE-SEM</td>
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<td>G</td>
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<td>HaCaT</td>
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<td>HBSS</td>
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<td>HTS</td>
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<td>ISIS</td>
<td>Intelligent Schematic Input System</td>
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<td>PDMS</td>
<td>Polydimethylsiloxane</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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</tr>
<tr>
<td>PEG</td>
<td>Polyethylene Glycol</td>
</tr>
<tr>
<td>PFPE-PEG</td>
<td>Perfluoropolyether - Polyethylene Glycol</td>
</tr>
<tr>
<td>PVC</td>
<td>Polyvinyl Chloride</td>
</tr>
<tr>
<td>PWM</td>
<td>Pulse Width Modulation</td>
</tr>
<tr>
<td>Q</td>
<td>Airflow Rate</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<td>RPM</td>
<td>Revolutions Per Minute</td>
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<tr>
<td>SD</td>
<td>Standard Deviation</td>
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<tr>
<td>SEI</td>
<td>Upper Secondary Electron Imaging</td>
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<td>United Kingdom</td>
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<td>US</td>
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<td>USA</td>
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<td>UTHM</td>
<td>Universiti Tun Hussein Onn Malaysia</td>
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<tr>
<td>V</td>
<td>Volt</td>
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<tr>
<td>Vdc</td>
<td>Volt Direct Current</td>
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<tr>
<td>wt/v</td>
<td>Weight per Volume</td>
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LIST OF APPENDICES

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CHAPTER 1

INTRODUCTION

1.1 Research background

Monolayer cultures in plastic vessels are routinely used in biological studies. However, the use of two-dimensional (2D) cell models for cell biological studies has its limitations [1, 2]. In 2D culture, the proliferation, differentiation, gene and protein expression, functionality and morphology of cells is considerably different from their physiological origin in vivo [3]. By contrast, the three-dimensional (3D) cell culture creates an artificial environment where cells are permitted to grow or interact with its surroundings. 3D cell culture is believed to have a better approximation to the tissue model for cell and tissue research because it restores specific biochemical and morphological features similar to the corresponding tissue in vivo [4]. In 3D cell culture, the connections between cells are more native-like and the behaviour of cells is more reflective of in vivo cellular responses [3, 5].

Regenerative medicine or biotechnology for creating living functional tissues in vitro is urgently needed for repair or replacement of damaged organs [6], application in cell culture and tissue engineering [7], pharmacological testing and bioengineering fields [8]. Microencapsulation is an intensive research area to create cell and tissue model for rehabilitation of functional tissues [9] and therapeutics purpose [10, 11]. It is a technique which encloses cells within a membrane or shell. It has been widely studied since 1960s [12]. A microcapsule is a hollow chamber with diameters in the range of a few micrometers to several thousands of micrometers [13, 14]. The semipermeable
membrane of the microcapsule can facilitate the transportation of proteins, deoxyribonucleic acid (DNA), and drug and allows the diffusion of oxygen, nutrients, therapeutic products and wastes, while blocking the entry of antibodies and immunocytes [15]. In tissue transplantation, microcapsules segregate cells from the surrounding tissue to protect the implanted cells from the recipient’s immune system [16]. Therefore, cell encapsulation in biocompatible and semipermeable biopolymeric membranes is an effective method to overcome rejection of the implanted organ [17].

There are various types of biopolymer such as agarose, collagen, alginate, chitosan and gelatin that are widely applied for encapsulation of cells [14, 18]. These materials are different in polymerisation process and hence this consideration greatly influences the design of the microencapsulation system. Among them, alginate is the most commonly used biopolymer for encapsulation of living cells because of many advantages it offers [19, 20]. Alginate is a naturally derived polymer, biocompatible in vitro and in vivo, with excellent biodegradability and provide rapid gelation process in the presence of divalent cations at room temperature [21]. Indeed, alginate has been employed for encapsulating cells and tissues to be transplanted into human body, as it is biocompatible to both the host and the enclosed cells [22]. Furthermore, alginate has been studied extensively and it is currently recognised as a clinically ready application material by the United States Food and Drug Administration (US FDA) [15, 23].

A few methods had been developed for the microencapsulation of cells such as simple dripping [24, 25], micromolding [26, 27], extrusion [28], microfluidic device [29, 30], electrostatic droplet generation [31-33], coaxial air-flow [34-36], vibration [37] and jet cutting techniques [38-40]. For simple dripping technique, the diameter of the capsules produced is usually ranging between 600 and 1000 µm [41]. This technique is used to produce microcapsules that does not involve with chemical or mechanical treatment. Other techniques such as microfluidic, micromolding and electrostatic dropping could produce smaller size of microcapsules ranging from 200 to 600 µm [34] but these techniques are considered harsh because involvement of the organic solvent, oil phase, high voltage and ultra-violet treatment to produce the microcapsules. The requirement for post-processing treatment due to the harsh generation techniques may be
threatening the survival rate of the living organism encapsulated in the microcapsules [8, 28, 42].

Amongst previous methods discussed [26-28, 30-33, 37-39, 43], aerosol atomisation technique is a simple and efficient method to generate microcapsules with well-controlled size and shape without the use of harsh chemicals [19, 36, 44]. In this study, an electronic aerosol atomisation system had been developed for the generation of 3D human keratinocytes (HaCaT) and oral squamous cell carcinoma (OSCC) cells (ORL-48) in microencapsulations of calcium alginate that leads to the growth of 3D microtissues in vitro.

1.2 Problem statement

Alginate based capsules can be generated by simply extruding droplets of sodium alginate solution from a syringe needle and the droplets are immediately allowed to polymerise in the calcium chloride bath. However, the simple dripping technique usually produced large diameter capsules of alginate in millimeter, that were recognised as unsuitable for medical and biotechnological applications [37, 45]. The size of the droplet is mainly dependent on the orifice diameter [46] and dripping can only be achieved when the extruded droplet’s of alginate continue to grow until its mass overcomes the surface tension at the tip of the needle [47]. Because of this limitation, other approaches have been developed to create microcapsules with smaller diameter [43]. Smaller alginate capsules in micron size are desirable because this range of microcapsules can equilibrate rapidly across the ultrathin membrane with larger surface to volume relationship, and hence provide better transport of gases and nutrients for the encapsulated cells [48]. Generation of alginate droplets by the electrostatic and JetCutter technique were shown to decrease the size of the droplets or capsules compared to normal dripping [24]. However, these techniques required sophisticated high voltages or strong electric fields, complex and bulky design of devices, respectively, that might be of high demand of energy and time during the fabrication of microencapsulation system [39, 49-51]. Microcapsules formed based on microfluidic emulsion technique are covered with oil and hence post-processing treatment is required to remove the oil film
for application in cell microencapsulation [52]. This is because the oil layer could block the exchange of gas and nutrient to the cells in the microcapsules. Involvement of harsh treatment to remove the oil film causing the cells in the microcapsules exposed more to the divalent ions or solvents which may present threats to the survival rate of the cells [8, 28]. Hence, the simpler the production process (without harsh and post-processing treatment), the less threat to the cells whilst ensuring cells to proliferate in the encapsulations.

In this thesis, an electronic aerosol atomisation system is proposed to generate the desired size of calcium alginate microcapsules for the microencapsulation of cells. Although the aerosol atomisation method has been developed previously [53, 54], but the microcapsules size was ranging from 10 to 40 µm which is too small and not suitable for cells encapsulation. Current applications (air jets, fuel injection and spray coating) based on aerosol atomisation technique required high air flow rate (50 - 600 l/min) and large volume (millilitre) of solution to create small beads size (approximately 1 - 3.5 µm) [55]. Thus, an adjustable electronic aerosol atomisation system employed for this research was designed to produce different airflow rates (0.2 - 0.5 l/min), in which it can be used to disperse small volume of cells-alginate suspension (microlitre) and to generate larger size of microcapsules with controllable size (range in 80 - 360 µm) which is suitable for cells encapsulation. Instead of using compressed air from a gas cylinder [19, 36] which is costly, the electronic aerosol atomisation system presented a different approach in the generation of airflow by using a direct current (dc) air pump. Moreover, this research applied OSCC and HaCaT cell lines for the microencapsulation that had not been reported previously. Oral cancer is the most common disease and it is a silent killer in the developing world, particularly in Southeast Asia country [56, 57]. Although the etiological factors of oral cancer are well established, the mechanism developed has rarely been studied and it is not well understood [56, 57]. In vitro microtissues models of OSCC could support the cancer research. Therefore, development techniques for generation of OSCC and HaCaT microtissues are essential. The OSSC (ORL-48) and HaCaT cells encapsulated are expected to grow into microtissues models that have applications for pharmacology study and preliminary prediction performance of their efficacy in therapeutic strategies.
1.3  Aim

The aim of the research is to develop an electronic aerosol atomisation system to generate calcium alginate microcapsules that are size controllable and able to encapsulate cells that leads to the growth of 3D microtissues.

1.4  Objectives

The following research objectives were established to achieve the aim. The objectives for this research are:

a) To develop an electronic aerosol atomisation system for generating calcium alginate based microcapsules of cells.
b) To encapsulate human Keratinocytes (HaCaT) and OSCC cell lines (ORL-48) using microcapsules of calcium alginate to form 3D cells.
c) To characterise the biophysical properties of calcium alginate microcapsules and the 3D microtissues produced.

1.5  Scopes

The four scopes of the research work are as follows:

a) Development and characterisation of an electronic aerosol atomisation system to generate microcapsules.
b) Synthesis of calcium alginate microcapsules with a diameter ranging from 200 to 300 µm, as the thickness of human epidermis by using an aerosol atomisation system.
c) Determine the extrusion rates and airflow rates of the aerosol atomisation system to generate appropriate size of microcapsules for cell encapsulation.
d) Encapsulation of HaCaT and OSCC (ORL-48) cells using calcium alginate to form 3D cells, monitor their growth in the encapsulation and investigate their biophysical properties.

1.6 Thesis contribution

The main contributions of this thesis are:

a) Electronic aerosol atomisation system with controllable airflow rate

The aerosol atomisation system has revived previous cell encapsulation techniques with no post-treatment process, no complex fabrication design of nozzle or high voltage requirement that would affect the cell survival rate in the alginate microcapsules [8, 28, 29, 42, 58].

b) Round shape and suitable size of 3D cells generated for the application

The findings obtained from the aerosol atomisation system have contributed to the understanding of how alginate is involved in driving the growth of both HaCaT and ORL-48 microtissues whereby the microcapsules and 3D cells generated were round shape and in consistent size [8, 30].

c) Encapsulate new cell lines of HaCaT and ORL-48

This is the first demonstration of microencapsulation of HaCaT and ORL-48 using calcium alginate microcapsules to be applied as a cell model for cancer research.
1.7 Thesis outline

Chapter 1 introduces the overview of this project with technology and technique of microencapsulation. The problems of the current 3D cell encapsulation technique were discussed, followed by the problem statement, aims, objectives, scopes, thesis contribution and thesis outline.

Chapter 2 consists of the review of the essential background study information in understanding the current body knowledge of microencapsulation and the latest development or technique developed in the field associated with the research topic.

Chapter 3 presents the methodology used to develop the electronic aerosol atomisation system and technique to produce 3D cells based on the calcium alginate microencapsulation. Calcium alginate microencapsulation to generate 3D cell technique, the development of an electronic aerosol atomisation system, the programming of microcontroller of the air pump, the circuit design and simulation of the aerosol atomisation system, the procedure in preparing the cells and calcium alginate for microencapsulation and the biophysical properties characterisation of the microcapsules and microtissues were discussed.

Chapter 4 unveils the performance of the aerosol atomisation system based on the pulse width modulation (PWM), potentiometer voltage and the effects of airflow rate and extrusion rates to the size of microcapsules. The biophysical properties of microcapsules generated were assessed using the optical microscopy, fourier transform infrared spectroscopy (FTIR) and field emission-scanning electron microscopy (FE-SEM). The growth of 3D HaCaT and ORL-48 cells into microtissues were monitored using inverted phase contrast and fluorescence microscopy. Nonetheless, the results of the biophysical properties of the microtissues formed were reported and discussed.

Chapter 5 summarises the problem statement that have been solved, the objectives that have been achieved and the future works to enhance this research.
CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

This chapter discussed and explained the background knowledge and information of cells and tissues, types of epithelial cells applied for microencapsulation, rationale of growing 3D cells, microencapsulations, applications of microcapsules, microencapsulation techniques, biopolymers used for fabrication of microcapsules for cell microencapsulation and the review on microscopy and spectroscopy techniques applied in this research.

2.2 Cells and tissue

The basic building blocks of all living things is cell [59]. Cells provide structure for human body, take in nutrients that are consumed, convert it into energy, and use them to carry out specialised functions. Organelles are specialised structures that perform important cellular functions within the cell. Human cells contain nine major organelles such as the cytoplasm, cytoskeleton, endoplasmic reticulum (ER), golgi apparatus, lysosomes, mitochondria, nucleus, plasma membrane and ribosomes as shown in Figure 2.1.
Tissue is structural organisation of cells with similar or identical specialised characteristics, contributing to the performance of a specific function. Tissues are parts of organs that provide numerous functions of organs necessary to maintain biological life. In humans, there are four basic types of tissue, which are epithelial, connective, muscular, and nervous tissues (Figure 2.2). Epithelial tissue covers the body surface and forms the lining for most internal cavities. The major function of epithelial tissue includes protection, secretion, absorption, and filtration. The skin is an organ made up of epithelial tissue which protects the body from harmful microbes [61]. Cells of the epithelial tissue have different shapes. Connective tissue is tissue that supports and binds other tissues. It consists of connective tissue cells embedded in a large amount of extracellular matrix.
2.2.1 Extracellular matrix (ECM) and cell adhesion

All cells in solid tissue are surrounded by extracellular matrix (ECM). ECM is composed of proteins and polysaccharides. In animal cells, the ECM surrounds cells as fibrils that contact the cells. Cells are linked directly to each other by cell adhesion molecules at the cell surface. ECM provides mechanical support [63], a biochemical barrier [64], a medium for extracellular communication [65], cell matrix adhesion [66], and adhesion matrix for cell migration [67-69] during cell development.

Adhesion of cells to the ECM is key to the regulation of cellular morphology, migration, proliferation, survival, and differentiation [70]. These functions are essential during development, maintenance of tissue architecture and the induction of tissue repair. Integrin are the predominant receptors that mediate cell adhesion to the ECM proteins [71, 72].

Attachment of cells to ECM components induces clustering of integrin on the cell surface [73]. The cytoplasmic portions of the clustered integrin then function as a
platform for the recruitment of cellular proteins and signaling proteins to the inner surface of the plasma membrane, where they form structures called focal adhesions (FA) (Figure 2.3 (a)) [74]. The FA provide strong linkages to the actin cytoskeleton mediated by integrins to connect cells firmly to the ECM [75].

Cells adhere to the ECM via integrins that function as a heterodimer that composed of subunits alpha (α) and beta (β) transmembrane linked to cell cytoskeleton actin microfilaments via talin and vinculin [76]. Talin is a main regulator of the initial process of FA assembly [77]. During the initial step of FA formation, the binding of talin to integrin stabilises the ligand-induced clustering by mediating crosslinking of integrins with vinculin and α-actinin (Figure 2.3(b)) [78].

Figure 2.3: Cell adhesion to the ECM. (a) Suspended cells adhere to the surface of ECM via integrins (b) The structures of actin cytoskeleton, focal adhesion complexes, integrin receptors, and adhesion proteins to form cross-linked platforms
2.3 Epithelial cells and skin

HaCaT and OSCC (ORL-48) are non-cancer and cancer epithelial cells, respectively. The microtissue models for epidermis and oral cancer cell study are scarce. Hence, the growth of both cell types into biomimetic microtissues would provide value in tissue implant [79], pharmacology [80] or even cancer therapeutic drugs study [11].

2.3.1 Human keratinocyte cell lines (HaCaT)

The epidermis is a squamous epithelium that forms the protective layer of the skin. It consists of renewing tissue with the main cell type (keratinocytes), superpositioned and organised into four histologically distinct cellular layers: stratum corneum, stratum granulosum, stratum spinosum and stratum basale [81]. The HaCaTs have a close similarity in functional competence to normal keratinocytes [82]. HaCaT is a spontaneously immortalised and transformed aneuploidy immortal keratinocyte cell line from adult human skin [83]. The naturally immortalised human HaCaT cell line can be grown in culture vessel (Figure 2.4) for long periods of time [84]. This cell line has been widely used for studies of skin biology, differentiation and scientific research as a paradigm for epidermal cells [84-86]. HaCaT grew in the form of monolayer and adherent to the culture dish easily. Under typical culture conditions, HaCaT cells have a partially to fully differentiated phenotype due to the high calcium content of both standard media and fetal bovine serum. HaCaT cells are used for high differentiate and proliferate capacity in vitro [87]. HaCaT cells drastically reduced tissue regeneration compared to normal epidermal keratinocytes [88, 89]. The deficiency in HaCaT cells were not due to the permanent loss of differential functions and can be solved by the addition of growth factors [84].
2.3.2 Oral squamous cell carcinoma cell line (ORL-48)

Oral cancer is defined as malignant lesion within oral cavity. Most cancerous oral cells originate from the oral squamous epithelium cell which is the primary surface structure of the lips and mucous membrane of the oral cavity [90]. OSCC has been histologically characterised as irregular nests, columns or malignant epithelial cells [91]. Abnormalities of oral cancerous cells are believed to be associated with several consecutive genetic mutations [92]. By clonal selection of viable cells which have accumulated genetic damages, normal mucosa cells ultimately evolve into malignant mucosa cells over an indefinite period [93].

ORL-48 is one of the OSCC cell lines derived in Cancer Research Malaysia. ORL-48 was surgically explanted specimens obtained from untreated primary human oral squamous cell carcinomas of the oral cavity [56]. It was derived from a female donor patient at the age of 79 years old having cancer tumour in the mouth and gum [56]. ORL-48 cell lines grew in the form of monolayers (Figure 2.5) with the population doubling times ranging between 26.4 and 40.8 hours and they are immortal [56].
2.4 Rationale of growing 3D cells

Sub-culturing monolayer of cells in plastic vessels is a routine procedure for cell biology study. However, the validity of using such a 2D cell model for cell biology or pharmacological study is controversial [1, 2]. Cells grown in monolayer proliferate involuntarily due to the contactless spreading of cells and it has been shown to produce limited amount of extracellular matrix proteins [94]. The cell behaviour such as proliferation, differentiation, gene and protein expression, general cell function and morphology is considerably different from their physiological origin in vivo [3]. In contrast, 3D cell culture creates an artificial environment in which biological cells are allowed to grow and interact with its surrounding environment in three dimensional. 3D cell culture is also proven to have better approximation to the tissue model for cell and tissue research because it reconstructs specific biochemical and morphological features similar to the tissue in vivo [4]. The connections between cells are more native-like and the cellular behaviour is more realistic.
2.5 Methods for culturing microtissues

3D cell culture methods are commonly accepted as more physiologically relevant methods and are believed to improve prediction of drug development process [95, 96]. There are several methods for culturing cells into 3D microtissues, which involved scaffolds, matrices (scaffold-free), gels or hydrogels and bioreactor as listed in Table 2.1. Scaffold based method is available in variety of materials with different porosities, permeabilities and mechanical characteristics designed to mimic the *in vivo* ECM of the specific tissues [1]. Whereas, microtissue culture using scaffold-free platforms do not contain added biomaterials or ECM. Cells grown and organised with their own generated ECM [96]. Gels or hydrogels culturing method aim to mimic the ECM and it has a soft tissue-like stiffness [97]. Cells can be cultured directly on the hydrogels (agarose, collagen and alginate) to form microtissues [98-100]. This method can be combined with other methods, such as scaffolds and microchips. The most ideal 3D cell culture method for high volume cell production and in vitro tissue engineering applications are the bioreactors method [101]. Microtissues cultured by using bioreactor method allows circulation of nutrients and removal of wastes within the reactor.

Table 2.1: A summary of 3D cell culture methods for culturing 3D microtissues

<table>
<thead>
<tr>
<th>Methods</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>Applications</th>
<th>Reference</th>
</tr>
</thead>
</table>
| a) Scaffolds     | • Large variety of materials possible for desired properties  
• Customisable  
• Co-cultures possible  
• Medium cost | • Possible scaffold-to-scaffold variation  
• May not be transparent  
• Cell removal may be difficult  
• High-throughput screening (HTS) options limited | • Basic research  
• Drug discovery  
• Cell expansion | [102-105]   |
Table 2.1 (continued): A summary of 3D cell culture methods for culturing 3D microtissues

<table>
<thead>
<tr>
<th>Methods</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>Applications</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>b) Matrices</td>
<td>• No added materials</td>
<td>• No support or porosity</td>
<td>• Basic research</td>
<td>[96, 106, 107]</td>
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<tr>
<td>(Scaffold-free)</td>
<td>• Consistent spheroid formation (control over size)</td>
<td>• Limited flexibility</td>
<td>• Drug discovery</td>
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<tr>
<td></td>
<td>• Co-cultures possible</td>
<td>• Size of spheroid limiting</td>
<td>• Personalised medicine</td>
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<td></td>
<td>• Transparent</td>
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<td></td>
<td>• HTS capable</td>
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<td></td>
<td>• Compatible with liquid handling tools</td>
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<tr>
<td></td>
<td>• Inexpensive</td>
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<td>• Microfluidic</td>
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<td>• Hanging Drop Microplates</td>
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<td>• Microarray</td>
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<tr>
<td>c) Gels / Hydrogels</td>
<td>• Large variety of natural or synthetic materials</td>
<td>• Gel-to-gel variation and structural changes over time</td>
<td>• Basic research</td>
<td>[103, 104, 108]</td>
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<tr>
<td></td>
<td>• Customisable</td>
<td></td>
<td>• Drug discovery</td>
<td></td>
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<tr>
<td></td>
<td>• Co-cultures possible</td>
<td>• Undefined constituents in natural gels</td>
<td>• Personalised medicine</td>
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<td></td>
<td>• Inexpensive</td>
<td>• May not be transparent</td>
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<td></td>
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<td>• HTS options limited</td>
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<tr>
<td>d) Bioreactor</td>
<td>• Several options available</td>
<td>• Cost</td>
<td>• Basic research</td>
<td>[101]</td>
</tr>
<tr>
<td></td>
<td>• High volume cell production</td>
<td>• HTS options limited</td>
<td>• Tissue engineering</td>
<td></td>
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<tr>
<td></td>
<td>• Customisable</td>
<td></td>
<td>• Cell expansion</td>
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2.6 Microencapsulation

Microencapsulation is a technology of packaging solids, liquids or gases to be encapsulated inside a tiny sphere, called microcapsule. A microcapsule is a small sphere with hollow chamber, micro-porous and semi-permeable wall around it [109]. Microencapsulation of active compounds is defined as a series of techniques whereby a
compound is coated or masked, to present it in the form of multiparticulate system. Microencapsulation process can be classified in terms of the microparticles or microspheres, based on their external morphology and internal structure (homogeneous or solid spheres) in micrometer range diameters [13, 34]. The material inside the microcapsule is referred to as the core, internal phase, or fill, whereas the wall is sometimes called a shell, coating, or membrane. Microcapsules can be classified into three basic categories as mono-core (also called single-core or reservoir type), poly-core (also called multiple-core) and matrix types (Figure 2.6). Mono-core is microcapsule which has a single hollow chamber within the capsule [110]. Poly-core is microcapsule which has a number of different size chambers within the shell [110]. Matrix type is of microparticle that has the active compounds integrated within the matrix of the shell material [110]. However, the morphology of the internal structure of a microparticle depends mainly on the shell materials and the microencapsulation methods that are employed [110].

![Figure 2.6: Different morphology of microcapsules. (a) Mono-core, Single-core or reservoir type, (b) Poly-core, Multiple-core, (c) and (d) Matrix type [110]](image)

The main functions of microencapsulation are to isolate, immobilise, stabilise and protect the core from its surroundings. Capsular membrane is to shield the material within and control the flow of materials across the membrane (Figure 2.7). It allows manipulating the diffusion rate of molecules leaving the microcapsule under specific conditions and protecting against degradation agents (humidity, light, pH and gases) [111]. Microcapsules are also performing as carriers for drugs delivery, removal of fragrances and other compounds to facilitate product handling and improve material
ability [34]. For microencapsulation of cells, the selection of a suitable encapsulating material is critical. The material is required to have appropriate porosity, which can facilitate the transport of nutrients, proteins, DNA, and drug while blocking attack of antibodies and immune cells [6]. The capsules must be mechanically stable and easy to handle. These requirements may be fulfilled by controlling the pore size and the thickness of encapsulating polymer membrane at microscale. Smaller pore size and thicker capsules membrane showed higher mechanical stability [112, 113]. The cell viability and metabolic status must be optimal if the encapsulated cells are in the order of hundreds micron in size [15].

Figure 2.7: Principle of immunoisolation by a microcapsule [15]

2.6.1 Application of microencapsulation

Microencapsulation offers the possibility to microencapsulate any substances in polymeric materials [6]. It is well known as the promising technique to fabricate novel
micro and nanostructured materials applied to a wide variety of applications. There are various applications of microcapsules that have already been introduced in the market.

One of the most important applications of microencapsulated products is in the area of crop protection [114, 115]. Polymer microcapsules, such as gelatin, serve as efficient delivery vehicles to deliver pheromone by spraying the capsule dispersion and protect the pheromone from oxidation and light during storage and release [116].

The major applications area of encapsulation technique is pharmaceutical or biomedical for controlled drug delivery [117-120]. Several drug delivery systems are replacement of therapeutic agents, gene therapy and vaccines use. The capsules are engineered to stick tightly to and even penetrate linings in the gastrointestinal track before transferring the drug contents over time into circulatory system or the targeted spot [119, 121]. Other than that, one of the most important medical applications of microencapsulation technology is to serve as a cushion or implant, such as breast implant [110].

Microencapsulation is used to overcome all the challenges in food industry by providing technology to incorporate minerals, vitamins, flavours [122] and essential oils in food [123]. Microencapsulation simplify the food manufacturing process by converting liquids to solid powder, decreasing production cost, help fragile and sensitive materials survive processing and packaging conditions and stabilise the shelf life of the active ingredient [124-126].

Microencapsulation also plays a crucial role in energy generation field. Hollow and multilayered plastic microspheres loaded with gaseous, deuterium, a fusion fuel, are used to harness nuclear fusion for producing electrical energy [127]. This fusion experiment process has been named as inertial confinement fusion (ICF) and it has been in use since 1980s [128].

Design and development of nanofiber-based microencapsulation as a novel materials by the inclusion of carbonaceous materials such as graphene, in aeronautics-grade matrixes (thermoplastics and thermoset resins) through the application of microcoatings and intermediate microlayers in sandwich panels and reinforcement of matrixes have been widely used in aeronautics application.
2.6.2 Technologies for microencapsulation of cells

The encapsulation of various materials and living cells inside capsules for different purposes in the pharmaceutical, chemical, food industry, agriculture, tissue engineering, biotechnology and medicine is of great importance. Microencapsulation of cells in hydrocolloid gel matrices is the technique that the cells are entrapped during gel formation, leading to spherical droplets containing cells. Some of the popular microencapsulation technologies generally produce the capsules of micron to millimeter size for microencapsulation of cells were listed in Table 2.2.

Table 2.2: Comparison of different microencapsulation technologies for encapsulation of cells

<table>
<thead>
<tr>
<th>Micro-encapsulation process</th>
<th>Cost</th>
<th>Complex design</th>
<th>High voltage</th>
<th>Material volume</th>
<th>Uniform Size (mm)</th>
<th>Post-processing /harsh treatment</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extrusion</td>
<td>Low</td>
<td>No</td>
<td>No</td>
<td>Large</td>
<td>No</td>
<td>2 - 10</td>
<td>[129-131]</td>
</tr>
<tr>
<td>JetCutter break-up</td>
<td>High</td>
<td>Yes</td>
<td>Yes</td>
<td>Large</td>
<td>Yes</td>
<td>&lt; 1</td>
<td>[39, 49, 132-134]</td>
</tr>
<tr>
<td>Spinning disc</td>
<td>High</td>
<td>Yes</td>
<td>No</td>
<td>Large</td>
<td>Yes</td>
<td>0.2 - 5</td>
<td>[135]</td>
</tr>
<tr>
<td>Micro nozzle array</td>
<td>High</td>
<td>Yes</td>
<td>No</td>
<td>Large</td>
<td>Yes</td>
<td>&gt; 0.5</td>
<td>[8, 19]</td>
</tr>
<tr>
<td>Vibration nozzle</td>
<td>Low</td>
<td>Yes</td>
<td>No</td>
<td>Medium</td>
<td>No</td>
<td>0.1 - 3</td>
<td>[37, 136, 137]</td>
</tr>
<tr>
<td>Coacervation/ emulsion method</td>
<td>High</td>
<td>Yes</td>
<td>No</td>
<td>Large</td>
<td>Yes</td>
<td>0.02 - 2</td>
<td>[130, 131, 138, 139]</td>
</tr>
<tr>
<td>Electrostatic droplet generation</td>
<td>High</td>
<td>Yes</td>
<td>Yes</td>
<td>Medium</td>
<td>Yes</td>
<td>&gt; 0.1</td>
<td>[31-33, 133]</td>
</tr>
<tr>
<td>Flicking</td>
<td>High</td>
<td>No</td>
<td>No</td>
<td>Medium</td>
<td>Yes</td>
<td>0.2 - 0.4</td>
<td>[97]</td>
</tr>
<tr>
<td>Air atomisation</td>
<td>Low</td>
<td>No</td>
<td>No</td>
<td>Medium</td>
<td>No</td>
<td>0.08 - 0.6</td>
<td>[35, 140, 141]</td>
</tr>
</tbody>
</table>
2.6.2.1 Extrusion, Jet break-up methods and spinning disc

Extrusion (Figure 2.8 (a)) is the most common methods widely used to produce microcapsules due to its ease, simplicity, low cost, gentle condition and high quantity of encapsulated cells. Jet break-up and spinning disc techniques are also originated from the extrusion method. In a basic extrusion technique, the alginate containing cells are extruded through a syringe needle as droplets into calcium chloride (CaCl\(_2\)) solution to be polymerised. The size and shape of the capsules were influenced by the aperture size of the needle, concentration of the CaCl\(_2\) solution and the surface tension of the CaCl\(_2\) solution. The basic extrusion technique produced capsules size ranging from 2 - 10 mm [129-131].

Jet cutter method is suitable to be used with high viscosity polymer solutions such as poly(vinyl alcohol) solutions [134]. In this technique, the mixture of cells-alginate suspension was forced through a nozzle to form liquid jet and then cut by a rotating cutting wire (Figure 2.8 (b)). The number of cutting wires, rotations speed of cutting tool and the infusion rate manipulates the size of the capsules.

For the spinning disc technique, the capsules are formed by infusing the cells-alginate suspension onto the high velocity spinning disc (Figure 2.8 (c)) due to the centrifugal force at the edge of the spinning disc, the droplets are formed and dropped into the CaCl\(_2\) solution to be polymerised. The size of the capsules is controlled by the rotating disc speed [142]. This method produces capsules with the size ranged from a few hundreds of micrometers up to several millimeters. In contrast to the jet cutting method in which, this method is suitable for fluid at low viscosity and it has a very high productivity.
2.6.2.2 Micro nozzle array and vibrating nozzle

Micro nozzle is a developed technique for microencapsulation in year 2000. In this technique, the cell-alginate suspension is flowed through silicon micro nozzle array and then cut off by the high stream of oil to form droplets [8]. The gel droplets drop into the oil stream that directs the flow of the droplets to a solution of positive ions (Figure 2.9 (a)). Due to the high flow pressure conditions, micro nozzle array are suitable to be used with high viscosity solution [144]. If this method is to be scaled up for large production, the cost of the oil and its disposability could be the limitations of this technique [145].

For vibration nozzle technique (Figure 2.9 (b)), the microcapsules are formed by oscillating and purging the mixture cells suspension through a nozzle into the hardening bath, resulting in size distribution of capsules as 0.1 - 3.0 mm in diameter [137].
2.6.2.3 Microfluidic device

Microfluidics device has emerged as a powerful platform for the generation of microparticles with tailored structure and properties [147-150]. This technique allows direct integration of different input fluids into the polydimethylsiloxane (PDMS) microfluidic channel as shown in Figure 2.10. The working principle of microfluidic to generate microcapsules is based on the emulsification of alginate solution.

Microcapsule fabrication methods based on microfluidics device may be classified into two major approaches, that are flow-focusing and T-junction capsule formation. The flow-focusing microfluidic approach, as shown in Figure 2.11, forms microcapsules by allowing a core fluid (cell-alginate suspension) to be surrounded by sheath stream (oil) flowing. In contrast, T-junctions microfluidic is designed to form microcapsules by permitting the core fluid to be swept away by one sheath stream in only one direction. A summary of the microfluidic emulsification technologies based on PDMS microfluidic chip design for both flow-focusing and T-junction capsules formation methods, used for the application of cell encapsulation were listed in Table 2.3 [6].
Figure 2.10: Illustrations of microfluidics system mechanism for microencapsulation [151]

Figure 2.11: Illustration of microcapsules fabrication methods based on microfluidics device. (a) Flow-focusing and (b) T-junction capsules formation [6]
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