DEVELOPMENT OF A NOVEL LIQUID CRYSTAL BASED 3D MICRO-TISSUES CULTURE TECHNIQUE AND MICROFLUIDIC VIBRATIONAL CLEANER

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To my beloved parents, sisters, my family, and all my beloved friends
LIST OF ASSOCIATED PUBLICATIONS AND AWARDS

Journal papers

1. A Scaffoldless Technique for Self-generation of 3D Keratinospheroids on the Liquid Crystal Surfaces
   Chin Fhong Soon, Kok Tung Thong, Kian Sek Tee, Arina Basyirah Ismail, Morgan Denyer, Yink Heay Kong, Patel Vyomesh, Sok Ching Cheong, Biotechnic & Histochemistry (under review)

2. The Effects of Culture Substrates and Media to the Behavior of Microtissues
   Kok Tung Thong, Arina Basyirah Ismail, Hatijah Basri, Kian Sek Tee, Chin Fhong Soon, ARPN Journal of Engineering and Applied Sciences (submitted)

Conference proceeding

1. The effects of enzyme to the dissociation of cells in monolayer and 3D microtissue on the liquid crystal substrate

2. Development of a Microfluidic Vibrational Cleaning System for Cleaning Microtissues
   Kok Tung Thong, Chin Fhong Soon, Arina Basyirah Ismail and Kian Sek Tee, The International Conference for Innovation in Biomedical Engineering and Life Sciences, Malaysia, 6-8th December 2015
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ABSTRACT

The conventional 2D culture technique used for producing monolayer of cells in the petri dish was widely practiced in the life sciences laboratory. However, this culture technique produced the organisation of cells that were far from the natural tissue and the outcomes for biological studies were not reliable. In this research, liquid crystal was exploited as a new culture technique to produce 3D micro-tissues of human keratinocyte cell lines (HaCaT). The development of 3D micro-tissues technique initiated with the optimisation of different cell density on the coated cholesteryl ester liquid crystal (CELC) substrate to obtain a higher number and larger sized of micro-tissues. The investigation of 3D micro-tissues physical characteristic included the analyses of cell–to–cell interaction, cell organisation, biochemistry element in micro-tissues, cell responses to enzymatic dissociation treatment, immunostaining and cell viability study of micro-tissues. A microfluidic vibrational cleaner constituted polydimethylsiloxane (PDMS) microfluidic chip and electronic system were developed to remove the residue liquid crystal from the extracted micro-tissues. Based on the cell monitoring and immunostaining, HaCaT cells were found with the ability to self-aggregate with adjacent cells and organised into microspheroid on liquid crystal substrate. This culture technique was able to maintain the cell viability of the micro-tissues around 80%. In addition, the micro-tissues presented potential to be re-cultured on polystyrene culture dish again. The liquid crystal residue on the extracted micro-tissues was effectively cleaned by the vibrational cleaner at a frequency of 148 Hz and acceleration of 0.89 Gms. The liquid crystal based 3D micro-tissue culture technique and the cleaning device was reliable to produce cleaned micro-tissues.
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<td>α</td>
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<tr>
<td>β</td>
<td>Beta</td>
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<tr>
<td>CO₂</td>
<td>Carbon Dioxide</td>
</tr>
<tr>
<td>O₂</td>
<td>Oxygen</td>
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<tr>
<td>μm</td>
<td>Micrometer</td>
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<td>2D</td>
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<td>5CB</td>
<td>4-Cyano-4'-pentylbiphenyl</td>
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<td>Ampere</td>
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<td>ATP</td>
<td>Adenosine Triphosphate</td>
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<td>CAD</td>
<td>Computer Aided Design</td>
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<tr>
<td>CELC</td>
<td>Cholesteryl Ester Liquid Crystal</td>
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<tr>
<td>CLA</td>
<td>Cholesteryl (L-lactic Acid)</td>
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<tr>
<td>CLS</td>
<td>Cell Line Services</td>
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<tr>
<td>cm</td>
<td>Centimeter</td>
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<tr>
<td>DAPI</td>
<td>4', 6-Diamidino-2-Phenylindole</td>
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<tr>
<td>DC</td>
<td>Direct Current</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
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<td>DSC</td>
<td>Differential Scanning Calorimetry</td>
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<td>D.W</td>
<td>Distilled Water</td>
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<td>ECM</td>
<td>Extracellular Matrix</td>
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<td>EDTA-trypsin</td>
<td>Ethylene Diaminetetraacetic-Acid Trypsin</td>
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<td>EFI</td>
<td>Extended Focal Imaging</td>
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<td>ER</td>
<td>Endoplasmic Reticulum</td>
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<td>FCS</td>
<td>Fetal Calf Serum</td>
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<td>FE-SEM</td>
<td>Field Emission-Scanning Electron Microscope</td>
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<tr>
<td>FTIR</td>
<td>Fourier Transform Infrared</td>
</tr>
<tr>
<td>GPa</td>
<td>Giga Pascal</td>
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<tr>
<td>G_{rms}</td>
<td>Acceleration Root Mean Square</td>
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<td>H&amp;E</td>
<td>Hematoxylin and Eosin</td>
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<td>Liquid Crystal</td>
</tr>
<tr>
<td>LCD</td>
<td>Liquid Crystal Display</td>
</tr>
<tr>
<td>MHz</td>
<td>Megahertz</td>
</tr>
<tr>
<td>MPa</td>
<td>Megapascal</td>
</tr>
<tr>
<td>mm</td>
<td>Millimeter</td>
</tr>
<tr>
<td>mA</td>
<td>Milliampere</td>
</tr>
<tr>
<td>mM</td>
<td>Milimolar</td>
</tr>
<tr>
<td>PCB</td>
<td>Printed Circuit Board</td>
</tr>
<tr>
<td>PDMS</td>
<td>Polydimethylsiloxane</td>
</tr>
<tr>
<td>PE</td>
<td>Polyethylene</td>
</tr>
<tr>
<td>PEGDA</td>
<td>Poly (Ethylene Glycol) Diacrylate</td>
</tr>
<tr>
<td>PLA</td>
<td>Poly (L-lactic Acid)</td>
</tr>
<tr>
<td>PWM</td>
<td>Pulse Width Modulation</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions Per Minute</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>SAMs</td>
<td>Self-assembled Monolayers</td>
</tr>
<tr>
<td>SD</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>SEI</td>
<td>Secondary Electron Image</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning Electron Microscope</td>
</tr>
<tr>
<td>UATR</td>
<td>Universal Attenuated Total Reflectance</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>UK</td>
<td>United Kingdom</td>
</tr>
<tr>
<td>USB</td>
<td>Universal Serial Bus</td>
</tr>
<tr>
<td>V</td>
<td>Volts</td>
</tr>
</tbody>
</table>
CHAPTER 1

INTRODUCTION

1.1 Overview

In routine two dimensional (2D) cell cultures, the cells proliferate into a thin monolayer of cell. The morphology and reality of monolayer cell is being questioned that is far from the native tissue, therefore three dimensional (3D) cell cultures is promoted to mimic in-vivo tissue. The general information of 3D cell culture is briefly explained in the background study. In this chapter, the problem statements draw out the shortcoming of the current 3D cell culture techniques and highlight the importance of 3D cell culture. The following section covered the objectives of study, scopes of study, thesis contributions and thesis outlines.

1.2 Background of study

Culturing cells in 2D or monolayer is a routine technique used in cell biology study. This technique is widely used in the study of cell proliferation, apoptosis, spread and transcriptional expressions. Culturing cells in monolayer is arguably an unnatural model that is far from the actual biological microenvironment in the tissue [1]. Majority of the publications in cell biology with their findings mainly relied on examining monolayer of adherent cells proliferated on polystyrene substrate (petri dish) or glass. This is far from the representation of the extracellular matrix within a tissue. In 2D cell culture, the cells are polarised and tightly coupled to the petri dish. In this state, cell-cell interaction is mainly concentrated at the periphery of the
flattened cell [2]. The surface of the flattened cell is barely exposed to the cell culture media. The spatial organisation of the cells is distorted as compared to the actual 3D in-vivo tissue. For in-vivo system, cells’ morphology and behaviour in the tissues are regulated by the mechanical cues, cell-cell communication and biochemistry release in a close encapsulation with other cells. However, in in-vitro system, cells can also be restored to the structures similar to those cells in the in-vivo system by using 3D cell culture techniques. This technique utilised biomaterials to provide cells with an environment mimicking the in-vivo system. 3D micro-tissues could be a more realistic model applied for drug screening, oncology study and new organ development. Biological responses measured from 2D cell culture arguably differ significantly from the 3D tissue model [3]. Therefore, it is more relevant to reproduce a growth environment that mimics the native tissue.

Cholesteryl ester liquid crystal (CELC) substrate is used to culture 3D micro-tissues in this research work. It is a new class of biomaterial that affinity to biological cell [4]. This biomaterial is sensitive to the environment. Its phases changed to thermotropic when exposed to heat and transformed into lyotropic phase when immersed into liquid. For cell culture application, lyotropic phase is highlighted.

This work proposed a new scaffold free method to culture 3D cell model on the cholesteryl ester lyotropic liquid crystal substrate and this method will produce a sustainable and simple model for repeating sub-cultures of 3D micro-tissues of human keratinocytes or keratinospheroids. 3D cell culture is useful for assessing cytotoxicity and kinetic of new drug but culture cells on the liquid crystal substrate causes the extracted micro-tissues to contain residue liquid crystals on its surface. Based on this issue, a mechatronic system will be developed to clean the micro-tissues for further analysis.

1.3 Problem statements

Cell is a highly adaptable and easily shaping biological entity. Based on the surrounding environment condition, cell changes its organisation and expressions [5]. Cells are usually cultured on stiff culture dish and the structural organisation of cells in 2D raised the question on the accuracy of this model for biological study. Due to the need for a more realistic model, various methods have been developed to culture cells in 3D construct. Some of the researches involved fabrication of
polydimethylsiloxane (PDMS) micromold to contain cells in close association [6], using soft biomaterial such as hydrogel to function as scaffolds for aggregating the cells [7], hanging drop technique to confined cells in liquid interfacial tension [8], and application of magnetic force [1] to aggregate cell into microspheroid or ellipsoid shape. For instance, cells cultured with hang drop technology which needed SureDrop™ inlet, GravityPlus™ plate and GravityTRAP™ plate to harvest the micro-tissues. In addition, magnetic cell levitation is a proposed scaffold free method to culture 3D cells but gold, magnetic iron oxide, and filamentous bacteriophage are present in the culture hydrogel for the purpose of cells aggregation via magnetic force. Most of these techniques stimulate growth of cells aggregation driven by restricted spatial or magnetic force. Previous presented techniques do not allows the observation of cells to grow based on the ability to self-organise; cell migration and stacking are not observed based on the confinement technique. Growing cells in 3D microstructure provide a model for cell biologists to study the mass transport of biomolecules such as understanding the effects of calcium alginate to the proliferation of cells in 3D [9]. The model could provide different perspective compared with the results found in 2D culture.

Therefore, a technique based on the growth of 3D cells on CELC was proposed. CELC was suggested for 3D cell culture because of its biocompatibility and suitable mechanical properties [11]. In order to obtain cleaned 3D micro-tissues, a microfluidic vibrational cleaner device was suggested to remove the liquid crystal from 3D micro-tissues surface.

1.4 Objectives of study

The main aim of the research is to develop a technique for culturing cells in 3D based on liquid crystal substrate. The following research objectives were established in order to achieve the main aim of this research:

a) To optimise suitable cell density to produce 3D micro-tissues and to determine the number of micro-tissues formed on the liquid crystal substrate.

b) To characterise the cell-to-cell interaction in micro-tissues and morphology of micro-tissues.

c) To conduct cell viability study on micro-tissues.
d) To develop a microfluidic device for cleaning the micro-tissues extracted from liquid crystals.

1.5 Scopes of study

Overall research is focused on develop a LC substrate method to culture 3D micro-tissues, investigate the characteristics of the micro-tissues and fabricate an electronic prototype to clean the harvested micro-tissues samples. The following scopes for this research work are justified to fit the domain of research as below:

a) Immortal human keratinocyte cell lines (HaCaT) with previous synthesised CELC substrate [10] will be adopted to culture the 3D micro-tissues in this research. The volume of suspension cells in 0.5, 1, 2, 3, 4, and 5 ml cell culture media will be selected to determine the optimum cell density for culturing the 3D micro-tissues.

b) Micro-tissues' surface structure and morphology study with field emission-scanning electron microscope (FE-SEM).

c) Several chemical drugs will be applied in order to facilitate the identification of cell organisation and the cell to cell interaction within the micro-tissues. Hematoxylin and eosin (H&E), 4', 6-Diamidino-2-Phenylindole (DAPI) fluorescence dye and Phalloidin (actin) fluorescence dye will be used to reveal the histological of micro-tissues, the DNA arrangement of cell and the actin protein distribution within the microspheroids.

d) Characteristic of drug response on monolayer cells and micro-tissues using the enzymatic dissociate buffer (EDTA-trypsin).

e) Cell viability study on dormant phase micro-tissues by using trypan blue exclusion assay and micro-tissues replating experiment on tissue culture treated culture dish.

f) Prototype design using computer aided design (CAD) software, SolidWorks 2013.

g) Development of microfluidic vibrational cleaner with manipulates control panel and water injection system.

h) Fabrication of microfluidic chip using adhesive vinyl as a replicated mould to produce PDMS based microfluidic chip.
i) Performance validation of the microfluidic vibrational cleaner system.

1.6 Thesis contributions

In this thesis, the experimental finding of a novel 3D micro-tissues culture method and the application of microfluidic vibrational cleaner revealed a new approach in the biological and engineering field. The contributions of this study are listed below:

a) Novel 3D micro-tissues culture method

LC based 3D micro-tissues culture technique is a breakthrough method of applying CELC as a substrate to proliferate the biological human skin cell line into 3D micro-tissues. This technique is able to produce 45 microspheroids per dish with a diameter of 159 µm on 72 hours of 3D micro-tissues culture.

b) Reduce the exploitation of animal model

Due to the close mimic of 3D micro-tissues with the in-vitro tissue model, scientist can reduce the use of animal model and the cost to maintain the animal houses.

c) New cleaning device

The continuous randomness vibration generated by the vibrational motor produced shock wave to mechanically remove the residue LC of micro-tissues. The cleaning effect of this device efficiently removed the residue LCs and the cleaning agent consumption was reduced by the microfluidic chip.
1.7 Thesis outlines

Chapter 1 introduced the comparison of 2D and 3D cell culture and the substrate material, CELC. The problems of the current 3D cell culture method were discussed, followed by the objectives, scopes, thesis contributions and thesis outlines.

Chapter 2 consisted of the review on the essential background information in understanding the current body of knowledge and the latest development in the field associated with research topic.

Chapter 3 presented the methodology used to develop the technique to culture 3D micro-tissues based on LC and development of a microfluidic cleaning device. LC based 3D micro-tissues technique, morphology observation of micro-tissues, quantification of the number of cells content in micro-tissues, the organisation of cells within the micro-tissues, enzymatic treatment of micro-tissues, the chemical properties of 2D cell and 3D micro-tissues, and the development of cleaning system were discussed in chapter 3.

Chapter 4 unveils the result of the 3D cell culture technique based on the LC. The optimum cell densities associated with size and quantities of the keratinospheroids formed were determined. The effects of different culture media on the generation of keratinospheroids were also determined. The biophysical properties of the keratinospheroids were analysed with FE-SEM, fluorescence staining, phase contrast microscopy and histological sectioning and staining. Nonetheless, the biochemical properties of the micro-tissues and 2D cells determined by fourier transform infrared (FTIR) spectroscopy were compared and distinguished. The performance of the microfluidic vibrational cleaner device was assessed based on the cross-polarising microscopy presented in chapter 4.

Chapter 5 summarised the objectives that have been achieved. The future works to enhance this research work is included in the final chapter.
CHAPTER 2

LITERATURE REVIEW

2.1 Overview

This chapter started with the literature review of eukaryote cell and the function of its organelles. The elucidation of cell’s activities with cytoskeleton and the secretion adherent protein were included in section 2.3. The nature proliferates environment of keratinocyte and it differentiates cycle in skin was discussed in section 2.4. The characteristic of 2D and 3D cell culture, previous development on 3D cell culture techniques and the applications of 3D cell in pharmacology were presented in section 2.5, 2.6 and 2.7, respectively. The relevant information of LC and the biomaterial carried out in this research was elaborated in section 2.8 and 2.9. The overview of the necessary instruments and the immunostaining method were discussed in section 2.10 and 2.11. A review of microfluidic chip and its application on cell engineering were included in section 2.12. This chapter was ended with the method to remove the LC and the effectiveness of mechanical vibration on biological cells.

2.2 Eukaryote cell and the role of the organelles

Eukaryote cell is a complex and large microorganism. The term eukaryote is derived from the Greek word which implicate “true nucleus”. This cell covers major life organism such as animal cell, plant cell, fungi and protest. Human skin epidermis HaCaT cell used in current research is one of the eukaryote cells. HaCaT cell
and eukaryote cell have similar cell organelle. The cell organelle consists of cell nucleus, cytoplasm, cytoskeleton and other. The cell structure of eukaryote cell is depicted in Figure 2.1 and the descriptions of the cell organelles are listed in Table 2.1.

![Diagram of eukaryote cell organelles](image)

**Figure 2.1: Diagram of eukaryote [11]**

**Table 2.1: The organelles of eukaryote [12]**

<table>
<thead>
<tr>
<th>Organelle</th>
<th>Description</th>
</tr>
</thead>
</table>
| Cell membrane | • It is made of phospholipids and protein.  
|             | • It serves as the cell’s boundary from its environment and selective permeate materials enter and leave the cells. |
| Cytoskeleton | • Networks of protein fibres that are involved in the organisation of the internal framework (shape), motility and support division of cell.  
|             | • Cytoskeleton is also responsible of the transportation of material around the cell (cytoplasmic streaming).  
|             | • There are three types of protein fibres:  
|             | a) Microfilaments named as actin filament. The thinnest fibre functions in cell division, amoeboid cell movement and shape the cell. It also serves as a track for myosin motor which carrying the ATP for cell.  
|             | b) Intermediate filaments, it provides the mechanical strength to cell and tissues.  
|             | c) Microtubules, an element to maintain the cell structure with microfilaments and intermediate filaments. |
| Centriole   | • Centriole is a set of short microtubules which take part in cell mitosis.  
|             | • It replicates itself into two and move to the opposite end of the cell. Subsequently, organizes and separate the chromosomes into two.
Table 2.1: The organelles of eukaryote [12] (continued)

<table>
<thead>
<tr>
<th>Cytoplasm</th>
<th>• A semi-fluid material contains enzyme for glycolysis (the first stage of respiration) and other metabolic reactions together with sugars, salts, amino acids, nucleotides, and everything else needed for cell to function.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ribosome</td>
<td>• Ribosome manufactures and translates the amino acids into protein by coded instruction from the nucleus.</td>
</tr>
<tr>
<td>Golgi apparatus</td>
<td>• It responsible in biochemically modify, package, store, and transport the proteins to the rest of cell from endoplasmic reticulum.</td>
</tr>
<tr>
<td></td>
<td>• It also creates the lysosome.</td>
</tr>
<tr>
<td>Lysosome</td>
<td>• Lysosome contains digestive (acid hydrolyses) enzymes that help to break down the unwanted chemicals (lipids, carbohydrates, and proteins), toxins, organelles or even the whole cell like a cleaner.</td>
</tr>
<tr>
<td>Endoplasmic Reticulum (ER)</td>
<td>• It is to transport all sorts of items around the cell.</td>
</tr>
<tr>
<td></td>
<td>• There are two types of ER:</td>
</tr>
<tr>
<td></td>
<td>a) Rough endoplasmic reticulum, an internal membrane system with ribosomes on its surface where proteins are assembled and manufacture the cell membrane.</td>
</tr>
<tr>
<td></td>
<td>b) Smooth endoplasmic reticulum, an internal membrane system to synthesis lipids and detoxification.</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>• Mitochondria convert food to adenosine triphosphate (ATP) as an energy producer.</td>
</tr>
<tr>
<td></td>
<td>• Through supply the energy, mitochondria involve in other processes such as cell signalling, cellular differentiation, cell death, cell cycle, and cell growth.</td>
</tr>
<tr>
<td>Nucleus envelope</td>
<td>• A double lipid bilayer membrane that surrounds the nucleus.</td>
</tr>
<tr>
<td></td>
<td>• On the surface of nucleus envelope with nuclear pores that control the message RNA (m-RNA) enter and send out the ribosome to ER.</td>
</tr>
<tr>
<td>Nucleus</td>
<td>• The biggest organelle in cell that contains the genetic material called chromatin (protein with DNA).</td>
</tr>
<tr>
<td></td>
<td>• Nucleus controls all the activity of cell such as growth metabolism and reproduction (mitosis).</td>
</tr>
<tr>
<td>Nucleolus</td>
<td>• A dark region in nucleus that making ribosomes and processing m - RNA.</td>
</tr>
<tr>
<td>Chromatin</td>
<td>• Granular macromolecules in cell which consist of DNA, protein and RNA.</td>
</tr>
<tr>
<td></td>
<td>• Chromatin package DNA into smaller volume to fit in cells, reinforce the DNA macromolecule to allow cell mitosis, avoid DNA damage, and control gene expression and DNA replication.</td>
</tr>
</tbody>
</table>

This section summarises the function of cell organelles for better understanding of its activity. Cell is a powerful self-organisation system, which can self-regulate to produce and store energy. It is responsible in delivering the genetic information to the reproduction system.

2.3 Cytoskeleton, integrin and extracellular matrix proteins

Cell to cell and cell to matrix attachment is a fundamental requirement to build a multicellular organism. The attachment of the cell is through complex adhesion
protein that bind cell to cell or cell to matrix. This activity involves the intracellular cytoskeleton (in particular, the microfilaments), extracellular matrix (ECM) protein and transmembrane receptor integrin as shown in Figure 2.2. Cytoskeleton is an organelle that involves the organisation of cell shape, mobility and division of cell. It requires to cooperate with integrin and ECM to response the biological action such as regulation of cell growth, differentiation, mobility and tissue pattern [13, 14]. Binding of cytoskeleton and ECM is performed by the ligand of integrin with α and β subunits chain. The proposal of binding is to probe the rigidity of the extracellular environment to mediate the adhesion protein. Integrin trigger the cell signalling for intracellular responses to remodel or degrade the extracellular matrix [15].

Figure 2.2: Mechanisms of cell-cell and cell-ECM interactions [16]
2.4 The relationship between keratinocytes and skin

Cell is the basic building block of all living organism unit of all living organic. Human is a living organism composed of various cells. Skin is the largest organ in the human and consists of multilayer of cellular structures. Epidermis constituted by four layers: basal (stratum basale), spinous (stratum spinosum), granular (stratum granulosum) and cornified (stratum corneum) layer as shown in Figure 2.3. Human keratinocytes found at the epidermis layer were the main composition of cells in the skin [17]. Keratinocyte stem cell starts division within the stratum basale and attached to the basement membrane by hemidesmosomes. Keratinocytes connect to the adjacent cells by desmosomes to produce lamellar bodies on stratum spinosum. The cells in the spinosum layer continuous with the presence of keratinocyte as a single cell type. As the cells proliferate and differentiate to the outer layer, keratinocytes lose their nuclei and cytoplasm in granular layer. This layer was filled with proteins that promote hydration and cross linking of keratin. The last stage of keratinocyte cells was cornified which consisted mostly of dead keratinocytes. Cornified layer will continuously shed and replaced by deeper strata cells [18]. Besides, this layer had higher stiffness compared to other layers in order to protect the underlying tissue from infection, chemicals and mechanical stress. Epidermis layer was the most superficial layer with a thickness of approximately 0.1 mm, and highest Young modulus approximately 1 MPa compared with the other skin layers [19].
2.5 Characteristics of 2D and 3D cell culture

Cell culturing is defined as the removal of cells from a living organism and growing it in an artificial environment. The artificial environment that promotes cells to grow consist of the essential nutrient supply (amino acid, vitamins, inorganic salts, carbohydrates and buffering agent), gases (in particular, O₂ and CO₂) and the physiochemical environment (pH, osmotic pressure and temperature). Cell culture provides a system to study the characteristic of cells for tissue engineering, biomedicine, pharmacology and veterinary science. Table 2.2 shows the milestone of cell culture from 1885 until present. The evolution of cell culture reflected the effort of previous researcher from 2D to 3D cell culture. Among them, Ross Granville Harrison was declared as the first who successfully worked with artificial tissue culture [21].
Table 2.2: Milestones of cell culture [22]

<table>
<thead>
<tr>
<th>Date</th>
<th>Event</th>
<th>Investigator</th>
</tr>
</thead>
<tbody>
<tr>
<td>1885</td>
<td>First tissue (chicken embryo) maintained in-vitro (for several days)</td>
<td>Wilhem Roux</td>
</tr>
<tr>
<td>1898</td>
<td>First human tissue (skin) maintained in-vitro (in ascitic fluid)</td>
<td>Ljunggren</td>
</tr>
<tr>
<td>1903</td>
<td>First tissue (salamander leucocytes) to be maintained for 1 month</td>
<td>Jolly</td>
</tr>
<tr>
<td>1910</td>
<td>First functional experiment (frog nerve fibre growth) and first general technique (use of lymph clot)</td>
<td>Ross Granville Harrison</td>
</tr>
<tr>
<td>1914</td>
<td>First culture cancer cells</td>
<td>Albert Ebeling and Joseph Losee</td>
</tr>
<tr>
<td>1922</td>
<td>First culture of epithelial cells</td>
<td>Albert Ebeling</td>
</tr>
<tr>
<td>1943</td>
<td>First continuous rodent cell line</td>
<td>Wilton Earle and George Gey</td>
</tr>
<tr>
<td>1951</td>
<td>First continuous human cancer cell line (HeLa)</td>
<td>George Gey</td>
</tr>
<tr>
<td>After 1951</td>
<td>Breakthrough of 3D cell culture</td>
<td>Multiple</td>
</tr>
</tbody>
</table>

Figure 2.4: 2D cells and 3D micro-tissues [23]
2D cell culture is considered as an early stage study on cell. The environment and characteristic of cell for 2D and 3D cell culture are totally different. Usually, 2D cell is cultured on stiff substrate such as culture flask or petri dish in different phenotype expressions and morphologically compared to 3D cell culture on soft substrate such as agarose gel or hydrogel [24, 25]. Figure 2.4 shows the different morphology within 2D cells and 3D micro-tissues. The information of 3D cell behaviour such as proliferation, differentiation, drug metabolism, expression (the gene, protein), general cell function, in-vivo relevance, morphology, response to stimuli, and viability are close to the in-vivo system [2]. A detailed comparison between 2D and 3D cell culture are listed in Table 2.3.

Table 2.3: Comparison of 2D and 3D cell culture characteristics [2, 26, 27]

<table>
<thead>
<tr>
<th>Definition</th>
<th>2D cell</th>
<th>3D cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells are grown on flat dishes made of polystyrene plastic that is very stiff and unnatural. The cells adhere and spread on this plastic surface and form unnatural cell attachments to proteins that are deposited and denatured on the synthetic surface.</td>
<td>Cells attach to one another and form natural cell-to-cell attachments. The cells and the extracellular matrix that they synthesise and secrete in three dimensions is the natural material to which cells are attached. It is flexible and close to the natural tissues. It is made of complex proteins in their native configuration and thus provides important biological instructions to the cells.</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Shape</th>
<th>Flattened monolayer.</th>
<th>Spheroids or ellipsoids.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Advantage</td>
<td>Simple</td>
<td>More complex and closer to the functions of native tissues than cells grown in 2D.</td>
</tr>
<tr>
<td>Environment</td>
<td>Approximately 50% of their surface area exposed to medium, approximately 50% exposed to the flat culture surface or intermediate, and a very small percent exposed to other cells.</td>
<td>Nearly 100% of their surface area exposed to other cells or matrix. It can exert forces on one another and can move and migrate as they do in-vivo. The cell-to-cell interactions in three dimensional cell cultures also include gap junctions which directly couple one cell to another.</td>
</tr>
<tr>
<td>Drug testing</td>
<td>Needs only to diffuse a short distance across the cell membrane to reach its intended target.</td>
<td>More realistic for mass transport of drug. A drug needs to diffuse across multi-layers of cells to reach the cells on the inside of a micro-tissue.</td>
</tr>
<tr>
<td>Growth Substrate</td>
<td>Stiff</td>
<td>Mimics natural tissue environment.</td>
</tr>
<tr>
<td>Architecture</td>
<td>Partially interact to adjacent cell and substrate.</td>
<td>Promotes close interactions between cells, extracellular matrix (ECM), growth factors.</td>
</tr>
</tbody>
</table>
2.6 Previous development of 3D cell culture techniques and applications

Cell in-vivo retain in a 3D viscoelastic microenvironment consist of cells and extracellular matrix. Comparatively, cell in-vitro was cultured in the culture flask which is indeed an unnatural environment for the attachment of cells. Therefore, the main framework of 3D cell culture techniques attempts to represent the 3D microenvironment that mimics the in-vivo system. The evolution of 3D cell culture was initiated by the discovery of colonial growth in agar gels in 1967. The morphology of the colonies was in microspheroid shape which was similar to the in-vivo cells. Due to this reason, various 3D cell culture methods were developed to proliferate 3D cells. Biomaterial, rotary instrument, confinement space, magnetic force and biodegradable polymer were implemented for 3D cell culture. The common point of these methods is simulating the condition of in-vivo system to the in-vitro cells. The evolutions of 3D cell culture are listed in Table 2.4.

Table 2.4: Evolution of 3D cell culture

<table>
<thead>
<tr>
<th>Date</th>
<th>Methods</th>
<th>Investigator</th>
</tr>
</thead>
<tbody>
<tr>
<td>1967</td>
<td>Culture 3D cell in agar gels</td>
<td>[28]</td>
</tr>
<tr>
<td>1971</td>
<td>Culture 3D cell in the spinner flasks</td>
<td>[29]</td>
</tr>
<tr>
<td>1972</td>
<td>Seed the cells on a three-dimensional formats with extracted extra cellular matrix (ECM)</td>
<td>[30]</td>
</tr>
<tr>
<td>1978</td>
<td>Coated agarose gels in a 4-well minidishe for 3D cell culture</td>
<td>[31]</td>
</tr>
<tr>
<td>1989</td>
<td>Culture and growth chondrocytes encapsulated in alginate beads</td>
<td>[32]</td>
</tr>
<tr>
<td>1991</td>
<td>Implement of rotating-wall vessels bioreactor to simulate the microgravity in-vivo condition</td>
<td>[33]</td>
</tr>
<tr>
<td>1998</td>
<td>Three dimensional synthetic biodegradable polymer scaffold for 3D cell culture</td>
<td>[34]</td>
</tr>
<tr>
<td>2004</td>
<td>Hanging drop technique to culture multicellular spheroids</td>
<td>[35]</td>
</tr>
<tr>
<td>2005</td>
<td>Culture 3D cell on mixture gelatin and chitosan gels architectures</td>
<td>[36]</td>
</tr>
<tr>
<td>2006</td>
<td>Micromold with collagen and polyethylene glycol (PEG)</td>
<td>[37]</td>
</tr>
<tr>
<td>2008</td>
<td>Magnetic reconstruction of three-dimensional tissues from multicellular spheroids</td>
<td>[38]</td>
</tr>
<tr>
<td>2008</td>
<td>A gel free 3D microfluidic cell culture system</td>
<td>[39]</td>
</tr>
<tr>
<td>2009</td>
<td>Hydrogels as Extracellular Matrix Mimics for 3D Cell Culture</td>
<td>[2]</td>
</tr>
<tr>
<td>2009</td>
<td>Paper platform for 3D cell culture</td>
<td>[40]</td>
</tr>
<tr>
<td>2011</td>
<td>Inducing 3D cell in PDMS microbubbles</td>
<td>[41]</td>
</tr>
<tr>
<td>2015</td>
<td>Liquid crystal substrate for 3D cell culture</td>
<td>[42]</td>
</tr>
</tbody>
</table>

Meanwhile, some of the 3D cell culture method were being commercialised such as HydroMatrix™ Peptide Hydrogel, 3D Petri Dish®, GravityPLUS™, alvetex® and NanoShuttle™-PL. These products promoted different concept on 3D cell culture method. HydroMatrix™ Peptide Hydrogel is a synthetic nanofiber
scaffold that is flexible to control and tailor the 3D architecture for 3D cell culture. In contrast, 3D Petri Dish® seeded the cell on soft agarose substrates for secreting the ECM to aggregate with the adjacent cell in the multiple microspheroid arrays. GravityPLUS™ hanging drop system promotes the cells aggregate via a meticulous cell culture platform. High porosity inert polystyrene scaffold that resemble microslabs of tissue was the selling point of alvetex®. The last product utilised the magnetic nanoparticle to aggregate the cell into microspheroids. With the help of those products, the research of 3D cell becomes more convenient and reliable. Further description of commercialised 3D cell culture product is listed in Table 2.5.

Table 2.5: Commercialised 3D cell culture product

<table>
<thead>
<tr>
<th>No.</th>
<th>Trade name</th>
<th>Strength</th>
<th>Weakness</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HydroMatrix™ Peptide Hydrogel [25]</td>
<td>- Precision and control of a synthesised matrix with the natural 3D architecture of highly crosslinked peptide hydrogel. - Able to control the flexibility of the 3D architecture, and tailor the structure to meet their individual needs.</td>
<td>- Hard to collect the mass cells. - The shapes of cells are nearly sheet form.</td>
</tr>
<tr>
<td>2</td>
<td>3D Petri Dish® [24, 43-63]</td>
<td>- The micromolds are autoclavable and reusable. - Make hundreds of spheroids of uniform size in a single pipetting step. - Able co-culture spheroids. - Harvest spheroids for RT-PCR and Western Blots without enzymes. - Scaffold-free and that maximise cell-to-cell interactions.</td>
<td>- Micromolds are limited base on the type of product.</td>
</tr>
</tbody>
</table>
Table 2.5: The commercialise 3D cell culture product (continued)

|   | 3 GravityPLUS™ [64, 65] | - Avoids any biomaterial to culture 3D cells.  
- A complete system to culture 3D cells from cell seeding until micro-tissues harvesting.  
- The transparent GravityTRAP™ plate is convenient for assays and imaging monitoring. | - Require compatibility with SureDrop™ and GravityTRAP™ technology.  
- Hard to change culture media.  
- Sensitive with vibration. |
|---|---|---|---|
|   | 4 Alvetex®: Polystyrene scaffold technology for routine three dimensional cell culture [66] | - Consistent structure  
- Based on existing cell culture material  
- Stable and inert  
- Adaptable to existing cell formats  
- Compatible with current methods of analysis | - Cells are embedded in the scaffolds of synthetic polymer.  
- Difficult to harvest the cells from the scaffolds for further analysis.  
- High porosity scaffold needed. |
|   | 5 NanoShuttleTM-PL [1] | - No effect on cell metabolism, proliferation, or inflammatory stress.  
- No special media or equipment required.  
- No interference with fluorescent microscopy and other experimental technique. | - Cells need to magnetise for static incubation overnight.  
- Released after 8 days |

The strength and weakness of 3D cell culture method are listed in Table 2.6. Some methods are based on the scaffold such as PDMS micromold [57], multizone paper platform [67] and hanging drop platform [68] while magnetic levitate cell [1] and cell encapsulated in calcium alginate microspheroid [69] are scaffoldless. Usually, scaffold based method is coated with a thin layer of biomaterial or pre-treatment on the biocompatible scaffold to mimic the native cell culture environment.
The function of scaffold is like a guiding mold or platform to shape the cell into micropsherooids. PDMS as a micro-mold for forming the construct to culture 3D micro-tissues is widely used because of its properties that provide chemical inertness, biocompatibility, high gas permeability, transparency, and high fidelity molding [41]. Micromold [57], microbubble [41], and microbowl [70] are similar PDMS scaffold techniques to grow 3D cells. On the other hand, cells will undergo a pre-treatment to form microspherooids via scaffoldless method. For instance, cells are required to magnetise itself overnight in order to magnetically form microspherooids under magnetic force.

<table>
<thead>
<tr>
<th>No.</th>
<th>Method</th>
<th>Strength</th>
<th>Weakness</th>
</tr>
</thead>
</table>
| 1   | Micromolded agarose gels [57] | - Form hundreds of spheroid with a piece of agarose gel micromolded.  
- Produce uniform micro-tissues shape and size. | - The post handling or extraction from the mold might be difficult because the cavity size is about 500 micrometer in diameter. |
| 2   | Mixture of synthetic and natural hydrogels scaffolds [2] | - Hydrogels mimics the native extracellular matrix to produce the micro-tissues.  
- Hydrogel provided better cell migration, cell-cell communication, and differentiation. | - The quantity of the cell in micro-tissues (B) is less |
| 3   | Paper platform for 3D cell culture [40, 67] | - Multiple layer of chromatography paper allows cells grow in multiple 3D geometries.  
- Mass transport of oxygen and glucose to cell are available. | - The destacking of chromatography paper may not be easy because of the thickness of each layer is around 200 μm. |

Table 2.6: Current 3D cell culture methods undergoing research and development
| 4 | Hanging drop technique [26, 68, 71] | • Less consumption of culture media.  
• Natural cell aggregate without any biomaterial. | • Sensitive with vibration.  
• A droplet of culture media caused it to dry up fast in incubator. |
|---|---|---|---|
| 5 | Magnetic levitation cell [1] | • Able to control the 3D cell shape.  
• Does not require a specific medium, engineered scaffolds, matrices or molded gels. | • Cell need to magnetise by the magnetic iron oxide contain in the hydrogels and incubated overnight. |
| 6 | Encapsulation of live cells in calcium alginate microspheres [69] | • Calcium alginate beads allowed viability of cell after encapsulate process.  
• Calcium alginate beads size can be vary by the material flow rates. | • Inhomogeneous structure of the cell containing alginate solution affected the droplet formation size. |
2.7 Application of 3D cell culture in pharmacology

3D cell culture is an active research area because of its potential application in biomedical engineering. For the purpose of pharmaceutical study of drugs a series of preclinical trials were conducted on non-human subject to ensure safe dose for the first-in-man study. Pharmaceutical study covers the efficiency of drug, toxicity, pharmacokinetic and pharmacodynamics. The toxicity test on 2D cell subjects along with in-vivo studies is gradually moved to 3D model cells. This is due to 3D cells which are found to be more precise than 2D cell on drug and cosmetics screening [72]. The synthetic 3D cell's characteristic was shown to be more similar to the native cell. As previously reported (Table 2.7), 3D cultured cells had found applications in anticancer cytotoxicity test [26], toxicity test on carcinoma cell [73], regenerative medicine [74], derma papilla transplant [6], and drug clinical trial [73]. These applications are more relevant testing platform before applied in the human physiological system.

Table 2.7: Application of 3D cell culture

<table>
<thead>
<tr>
<th>No.</th>
<th>Application</th>
<th>Method</th>
<th>Cell type</th>
<th>Finding</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Anticancer drug and hypoxia-triggered cytotoxicity tirapazamine assay on monolayer and 3D spheroids cell</td>
<td>Drug testing using 384 hanging drop array</td>
<td>A431.H9 cells</td>
<td>3D cells are more resistant on the inhibits cellular proliferation but less resistant on the core hypoxic.</td>
<td>[26]</td>
</tr>
<tr>
<td>2</td>
<td>3D liver tissue to assess toxicity of cadmium telluride and gold nanoparticles</td>
<td>Hydrogel inverter colloidal crystal (ICC) scaffolds</td>
<td>Human liver carcinoma cell line (HepG2)</td>
<td>Diminishing toxicity related to the tissue morphology and phenotypic change.</td>
<td>[73]</td>
</tr>
<tr>
<td>3</td>
<td>Regenerative medicine</td>
<td>Micro fabrication of microchip with organs on chip</td>
<td>Liver hepatocytes, airway epithelial cells, renal tubular epithelial cell, intestinal epithelial cells, osteoblasts, osteocytes, corneal epithelial cells, brain neurons</td>
<td>Proposed a novel concept of a human physiological system on a chip.</td>
<td>[74]</td>
</tr>
</tbody>
</table>
Table 2.7: Application of 3D cell culture (continued)

<table>
<thead>
<tr>
<th></th>
<th>Transplantable derma papilla</th>
<th>Patterned PDMS-based cell arrays, tissue culture polystyrene plates</th>
<th>Derma papilla and keratinocytes</th>
<th>Able to grow hair after grafting the functional DP tissues into the dorsal side of new born nude mice epidermis for 5 weeks.</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td></td>
<td></td>
<td></td>
<td>[6]</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Hair follicle-like hydrogel</td>
<td>Soft lithography of PEGDA hydrogels</td>
<td>Dermal and epithelial were seeded in different compartments of the micro structured mold of PEGDA hydrogel.</td>
<td>Decreased cell viability when culture up to 14 days which could be due to the toxicity of UV light polymerisation of PEGDA hydrogel.</td>
<td>[75]</td>
</tr>
</tbody>
</table>

2.8 Liquid crystal

LC was discovered by an Austrian botanist, Fredrich Reinitzer in 1888. He found that cholesterol ester of plants and animals had the phenomenon of double melting points. At 145.5°C, it melted from solid to a cloudy liquid and turned into a clear liquid at 178.5°C. He also observed the transition of colour behaviour when the sample cooled down, initially pale blue appeared as the clear liquid turned cloudy and subsequently a bright blue-violet was present as the cloudy liquid crystallised. He was unable to explain this unusual phenomenon by himself so he sent the sample to Otto Lehman, a German physicist expert in crystallisation properties of various substances. Through the observation under a heat control polarising microscope, Lehman concluded the opaque phases of cholesterol ester were a uniform phase with both liquids and solids properties as LC. After the announcement of LC, a French mineralogist, Georges Friedel proposed the classification of LC structural properties in 1922 [76].

The physical property of LC is intermediate between amorphous liquid and crystalline solid. This property is also named as mesophases where the molecules positional and orientational orders of LC are fixed in solid contrary to the orientation of molecules randomly tumbling in liquid. The feature of LC is its anisotropic property that changes its phase whiles the molecule is directed along different axes. The chemical structure of typical LC molecules is as shown in Figure 2.5.
This structure consists of two aromatic rings and linkage to terminal group and the side chain.

Besides that, the transition of LC phases also brings out different colour behaviour and texture of LC. LC phases and transition of mesophases can be identified by polarising microscopy and differential scanning calorimetry (DSC). The polarising light transmitted by the polarising microscope reflected the orientation of LC molecules and display its characteristic in colour gradient and texture as shown in Figure 2.6. However, real time monitoring data of the LC phase transitions under a heating chamber can be obtained by DSC.

Generally liquid crystal is divided into two either sensitive to the temperature or liquid solvent. Those having molecules with transition driven by heat are known as thermotropic LC, and those influenced by the solvent (typically water) are known as lyotropic LC.

![Figure 2.5: Molecular structure of typical liquid crystal [77]](image)

![Figure 2.6: Phase transitions in molecules and observed texture under polarisation microscope for the smectic A phase and nematic phase as an example [78]](image)
2.8.1 Thermotropic liquid crystal

The direction and position of the molecule are changed when LC is exposed to heat. Thermal motion varies the angle of the molecule from anisotropic LC to isotropic liquid as shown in Figure 2.7. Thermotropic LC can be classified into smectic, nematic and cholesteric phase. The transformation of thermotropic LC depends on the increasing temperature from crystalline to smectic followed by nematic or cholesteric phase and end with isotropic liquid as shown in Figure 2.7.

![Temperature](image)

Crystal Smectic Nematic Cholesteric Isotropic liquid

Figure 2.7: Transformation of thermotropic liquid crystal phases

Smectic phase is the first transition phase of thermotropic LC which occurs at low temperature compared to nematic and cholesteric. Smectic phase has three classifications: smectic A, smectic B and smectic C which determined the respective degree of translational order. Molecules aligned themselves parallel and organised into single layers. The positional order of molecules still remained when LC melts to form a smectic LC.

Nematic phase occurs at the second melting point. This phase is characterised by molecules that have no positional order but all the molecules are directed on average in a particular direction known as director, n. If this material receives higher
temperatures, the motion of the molecules transforms again to become an isotropic liquid.

Cholesteric phase is similar to the nematic phase but with the chiral structure. The chiral structure organised the molecules in layers with different plane. This structure is arranged helically and the director of the molecules is twisted along the z-axis normal to x-axis in 360°. Due to the helically structure, cholesteric phase LC reflected the selective colour and display the textures based on the homeotropic orientation caused by the temperature, pressure and electric and magnetic fields. Cholesteric phase has great potential uses as sensors, thermometer, fashion fabrics that change colour with temperature and display devices.

The colour reflection due to the interference of temperature or electric and magnetic field promotes the application of thermotropic LC in LCD [79] and thermometer [80].

2.8.2 **Lyotropic liquid crystal**

Lyotropic LC is a compound which has two immiscible hydrophilic head and hydrophobic tail that exhibit LC properties when exposed in solvent. It consists of amphiphile polar head and a hydrophobic tail in contrast to thermotropic rod-like molecule. Lyotropic LC also has several phase transition such as thermotropic LC. The transition of lyotropic LC phases depended on the amphiphile concentration of the solute material in the solvent. The progression transition of lyotropic phases from low to high amphiphile concentration is as shown in Figure 2.8. Lyotropic LC is a self-assembled structure which aggregate itself to specify arrangement when the amphiphilic concentration changes. The higher the amphiphilic concentration, the structure assemblies become more orderly. Usually in oil-water mixtures solvent, the molecules of lyotropic self-assembly in reverse where the amount of water is lesser and resides inside the micelle.
REFERENCES


