FUNDAMENTAL STUDY OF PULSE ELECTRIC FIELD EFFECTS ON HELA CELL CULTURED OVER EXTRACELLULAR MATRIX PROTEIN MICRO-PATTERNED SURFACE

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A thesis submitted in
fulfilment of the requirement for the award of the
Degree of Master in Electrical Engineering

Faculty of Electrical and Electronic Engineering
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AUGUST 2017

For my beloved Mother and Father

ACKNOWLEDGEMENT

I would like to take this opportunity to express my greatest gratitude to almighty Allah for His help and support during the course of life and the moment of truth.

I would like to express my appreciation and sincere gratitude to my supervisor, Associate Professor Dr. Muhammad Mahadi Bin Abdul Jamil for his continuous support, encouragement and endless patience towards completing the course of this master research. I am thankful for his aspiring guidance, invaluable construction criticism, and friendly advice during the project work. I am sincerely grateful to him for sharing his truthful and illuminating views on a number of issues related to the project.

I would like to express my appreciation to both of my beloved parents, my dad, Hj. Abd Rahman B. Abd Samat and my mom, Hjh. Rohaya Bt. Hashim for their tremendous patience, effort, support and encouragement during my master research. I really appreciate it. I would also sincerely like to thank my friends, Noor Hafizzatul Izzah Bt. Mat Harun, Nur Shazilah Bt. Aziz and Atikah Bt. Daud, for their support, encouragement and advice during my master and also to all the people who have contributed directly or indirectly for the accomplishment of this master research.

I would like to express my appreciation to UTHM, for the sponsor of my studies via GIPS (Vote: U167) until the completion of my studies. Last but not least, the Ministry of Higher Education for the sponsor of research materials and conference travel fund through Fundamental Research Grant Scheme (FRGS Vote: 1488). Thank you.

ABSTRACT

Electroporation (EP) is a method of controlling cell function by using pulses of electrical fields to create pores through cell membrane and causes other substance around it to be absorbed into the cell. This method has led to a variety of medical applications, particularly in cell studies. In this study, a high voltage of 2 kV/cm with pulse duration of 30 µs was applied on HeLa cell (human cervical cancer cell) to investigate the electroporation process. In addition, this study focused on the effect of protein coated surface, combined with the pulse parameter mentioned above, to look at its effect on HeLa cell when exposed to high voltage. Thus, will lead towards cell surface attachment factors interrogation plus the presence of electric field as the stimulator for an aggressive growth rate of the cells. This was achieved by using the micro contact printing (µCP) method. The result showed positive respond on the effect of EP on protein printed surface combination where HeLa cells were grown. The 50µm was chosen as the best-pattern size for cell alignment by using fibronectin. From the cell guidance study we could clearly see the cell responses on the protein patterned surface are much elongated in comparison to the control. In addition, the cells plated on this patterned surface were further investigated with electroporation technique, in order to see the effect of electroporation on the cancer cell proliferation and other cellular activities. The result shows that the cells aligned and elongated on fibronectin pattern with PEF than without PEF exposure. The combination of these two techniques will contribute towards understanding the cell surface interface and cell surface attachment factors which may lead towards a new method for guiding cell towards wound healing process.

ABSTRAK

Electroporation (EP) merupakan satu kaedah yang digunakan untuk mengawal fungsi sel dengan menggunakan denyutan medan elektrik bagi mewujudkan liang pada permukaan sel membran dan menyebabkan molekul lain yang berada di sekelilingnya diserap masuk ke dalam sel. Kaedah ini telah membawa kepada pelbagai aplikasi perubatan, terutamanya dalam kajian sel. Dalam kajian ini, voltan tinggi 2 kV / cm dengan tempoh denyutan 30 μs digunakan pada HeLa sel (sel kanser pangkal rahim) untuk mengkaji proses EP. Selain daripada itu, kajian ini memberi tumpuan kepada kesan permukaan bersalut protein digabungkan dengan parameter EP yang dinyatakan di atas, untuk melihat kesan ke atas sel HeLa apabila terdedah dengan voltan tinggi. Oleh itu, akan membawa ke arah faktor lekatan sel pada permukaan kajian ini ditambah dengan kehadiran medan elektrik sebagai perangsang untuk meningkatkan kadar pertumbuhan sel. Ianya dicapai dengan menggunakan kaedah *microcontact printing* (μCP). Hasil kajian menunjukkan tindak balas positif pada kombinasi kesan EP pada permukaan protein bercetak di mana sel HeLa telah diletakkan. Saiz 50 μm dipilih kerana saiz corak terbaik bagi penjajaran sel dengan menggunakan fibronectin. Daripada kajian bimbingan sel ini kita dapat melihat dengan jelas tindak balas sel pada permukaan protein bercorak dengan lebih memanjang berbanding dengan kawalan. Di samping itu, sel yang diletakkan pada permukaan bercorak ini telah dikaji pula dengan teknik EP, bagi melihat kesan EP pada percambahan sel kanser dan aktiviti sel lain. Hasil kajian telah menunjukkan bahawa sel sejajar dan memanjang pada permukaan *fibronectin* bercorak di bawah pendedahan PEF berbanding tanpa pendedahan PEF. Gabungan kedua-dua teknik ini akan menyumbang ke arah pemahaman antara interaksi permukaan sel dan faktor lekatan permukaan sel yang boleh membawa ke arah satu kaedah baru untuk membimbing sel ke arah proses penyembuhan luka.

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LIST OF SYMBOLS AND ABBREVIATIONS

µCP	Micro contact printing
CAD	Computer Aid Design
DNA	Deoxyribonucleic acid
EC magnetic Chamber	Perfusion type electrical stimulation magnetic chamber
ECM	Extracellular Matrix
EP	Electroporation
FBS	Fetal Bovine Serum Protein
FBS	Fetal Bovine Serum
FN	Fibronectin
PBS	Phosphate Buffered Saline
PDMS	Polydimenthylsiloxane
PEF	Pulsed electric field
Pen/Strep	Penicillin-streptomycin
RNA	Ribonucleic acid

SAMs	Self-Assembled Monolayers
TC	Type of Chambers
GAGs	Glycosaminoglycans

LIST OF PUBLICATION AND AWARDS

Journal:

1. **Nur Adilah Abd Rahman**, Mamman Hassan Buhari, and M. Mahadi Abdul Jamil, "An Overview: Investigation of Electroporation technique on cell properties cultured on Micropatterned surface." Jurnal Teknologi, Vol. 77, No. 6, Medical Engineering Vol. 1, Pg. 61-65, 2015.

2. Safyzan Salim, **Nur Adilah Binti Abd Rahman**, M. Mahadi Abdul Jamil, Mansour Youseffi, Morgan Clive Thomas Denyer, "Investigation of Electroporation Technique On Cell Properties Cultured On Self Assembled Monolayer.", Journal of Biological Sciences, Vol. 16, No.7, Pg.278-283, 2016.

Book Chapter:

3. Muhammad A. Milad Zaltum, **Nur Adilah Abd Rahman** and M. Mahadi Abdul Jamil (2016). Pulse Duration Effect on Growth Rate of HeLa Cells. Muhammad Mahadi Abdul Jamil. Biomedical Engineering Applications: Cell Engineering, Penerbit UTHM, 4; 47-56.

Conference Proceedings:

4. **Nur Adilah Abd Rahman**, M. Mahadi Abdul Jamil, "Investigation of Pulsed Electric field on cancer cell cultured on patterned surface." IEEE, International Conference on control System Computing and Engineering (ICCSCE, 2016), 25th – 27th November 2016, Batu Feringghi, Pulau Pinang, Malaysia.

5. **Nur Adilah Abd Rahman**, M. Mahadi Abdul Jamil, "Enhancement of cell migration on protein pattern surface with the assistance of Pulsed Electric field: Cell Guidance Study." ASIA International Multidisciplinary Conference (AIMC-2017), 1-2 May, Universiti Teknologi Malaysia, Johor Bahru, Malaysia, 2017**. (Presented)**

6. M. Mahadi Abdul Jamil, **Nur Adilah Abd Rahman**, "Enhancement of cell migration and proliferation rate with the assistance of Pulse Electric field: Cell Migration Study.", International conference on electrical and electronic engineering 2017 (ICE3-2017), 8-9 May, Pulau Langkawi, Malaysia, 2017. **(Presented)**

Awards:

7. Investigation of Electroporation Technique On Cell Properties Cultured On Self Assembled Monolayer. Safyzan Salim, **Nur Adilah Binti Abd Rahman**, M. Mahadi Abdul Jamil, Mansour Youseffi, Morgan Clive Thomas Denyer. The International Conference on Engineering Technologies & Entrepreneurship 2015 (ICETE-2015), 16th - 18th November 2015, Kuala Lumpur, Malaysia.

   ("BEST PAPER AWARD")

Research Grant Award:

8. Grant: Investigation of Electroporation Effect on Cell Properties Cultured on Micropatterned Surface. Geran Insentif Penyelidikan Siswazah (GIPS), 31st March 2016.

CHAPTER 1

INTRODUCTION

1.1 Background of study

Micro contact printing (μCP) has been developed since about 20 years ago. It is an impressive surface patterning technique in micron and nano scale. Surface science communities such as engineers and biologists have been focusing in μCP and therefore enriching the improvement of the μCP process itself. A μCP is a soft lithography that is used for the release of pattern on master polydimenthylsiloxane (PDMS) stamp to form patterns of self-assembled monolayers (SAMs) print on the surface of a substrate by conformal contact. In the original version of μCP, the micrometre-scale patterned for chemical modification of a large surface area was found by transferring different types of compounds using soft polymer stamp (Maksud M. I., Yusof M. S., and Abdul Jamil M. M., 2013). The capability to generate patterns of proteins and cells on surfaces are important for biosensor technology (Thomas C. A., 1972, Gross G. W. et al., 1995, Jun D. R. et al., 1998 and Mrksich M. and Whitesides G. M., 1995), for tissue engineering (Merritt M. V., Mrksich M. and Whitesides G. M., et al., 1997) and for fundamental studies of cell biology (Mrksich M. et al., 1996b, Singhvi. R. et al., 1994, and Chen C. S. et al., 1997). Tissue engineering is essential for the cells to be placed in specific positions to create organized structures or it called cell migration which is important in tissue formation and cell guidance. Thus, the placement of biological ligands at well-defined locations on substrates is required for certain

biological assays (Ravi S. K. et al., 1999, R. Singhvi R. et al., 1994, Chen C. S. et al., 1997, Lopez G. P. et al., 1993 and Mrksich M. et al., 1996b).

Patterning technique controls the size and shape of the cell that is attached to a surface, the chemistry and the topology of the substrate to which the cell is attached towards cell guidance. The technique is also particularly useful in understanding the effect of cell-material interface on the behaviour of cells (Singhvi. R. et al., 1994, Chen C. S. et al., 1997, Lopez G. P. et al., 1993, and Mrksich M. et al., 1996). Photolithography is a technique that has been used widely for patterning proteins and cells (Ravi S. K. et al., 1999). It can be used to produce patterns by photo ablating proteins preadsorbed to a silicon or glass substrate (Hammarback J.A. et al., 1985). In order to see the patterned samples using inverted microscope, transparent material such as glasses is used as a substrate. Polydimethylsiloxane (PDMS) is suitable for patterning on glasses because it shows a good adhesion on glasses and it can be peeled off smoothly from the surface (Kyoko A. et al., 2004). There are several methods on controlling wound repair and cell behaviour by using a mixture of topographic guidance and topographic/adhesive guidance signals such as micro contact printing techniques (Abdul Jamil M. M. et al., 2007).

Electroporation (EP) or electropermeabilization is a method to introduce molecules, or a method for increasing cell membrane permeability to molecules by applying high magnitude electric pulses (Chunlan J. et al., 2015). It has been used in several biotechnological and biomedical applications, such as the introduction of molecules into cells, cell fusion, tissue ablation, and also sterilization of water and liquid food. (Davalos R. V. et al., 2004). This method can be applied in 3 ways that is *ex vivo, in vivo*, and *in vitro* (Chunlan J. et al., 2015). Cell electroporation *in vitro* is used mostly for transfection by DNA introduction and microbial killing. *Ex vivo* electroporation provides the influence of cells that is reintroduced into the body to provide therapy. *In vivo* electroporation of tissues boosts molecular transportation through the tissues and into their constitutive cells (Weaver J. C., 2000). By applying an electrical pulse across cells, a variety of outcomes can be obtained ranging from no effect to a reversible electroporation which transiently permeablize cell membrane (Zaltum M. A. M., Adon M. N. and Abdul Jamil M. M., 2013) to irreversible electroporation (Sundararajan R. et al., 2014). This technique has become a widespread technique for loading cell with substances because it can be implement to

any cell type (Zaltum M. A. M., Adon M. N. and Abdul Jamil M. M., 2013, Dev S. B. et al., 2000).

Electrically induced transfer of material into cells and tissues present an opportunity for many new medical treatments and provide a valuable tool for the study of the basic structural and biochemical behaviour of cellular and intercellular system (David W. J. et al., 2004). This technique has been found to be an effective technique to overcome membrane barrier (Dev S. B. et al., 2000). Briefly, if a membrane with a high electrical resistivity surrounds a biological entity with a low resistivity, as in the case of a cell, an applied electrical field is enhanced dramatically in the membrane thickness. Consequently, the high field strength in membrane can lead to the formation of area with increased permeability, or better known as pores, which allows transmembrane transportation of molecules (Dev S. B. et al., 2000). Furthermore, if the pulse electric field applied is not too strong and the exposure set is not too long, the pores reseal in seconds to minutes after the exposure and the cells return to their normal activity (Lea R. et al., 2013). Electric field does not only affect excitable tissue such as muscle and nerves but also the non-excitable tissue, either thermally, by producing heat inside the tissue or inducing structural changes down to cellular membranes (Zaltum M. A. M., Adon M. N. and Abdul Jamil M. M., 2013).

1.2 Problem statement

Wound healing is a process of replacing devitalized and absent cellular structure and tissue layer. For a human adult, wound healing process is divided into 3 phases which are inflammatory, proliferative and remodelling. However, in certain cases, there are problems regarding wound healing due to age related factors such as inability to form a blood clot, poor inflammatory response, inability to produce new cells, regeneration of new tissue and infection. These problems were tackled by using protein, drugs and cytokines such as transformation growth factor. This could be costly and very much chemical therapeutic compounds dependent.

Micro contact printing is a quite useful method in several applications such as extracellular matrix patterning for cell adhesion molecule to promote cell attachment

for the cell assembly, growth and cell guidance. It is important for the cell to be directed towards specific locations in order to enhance the wound coverage and thus resulting in tissue regeneration. The patterning technique can control both the size and shape of the cell that attaches to the surface of the substrate. Therefore, in this research, the micro patterning technique is combined with the electroporation method in order to look at the feasibility of pulsed electric field effect in enhancing the growth rate of cell. The combination also demonstrates the effect on the guidance of cell potentially as an alternative to the usage of readily available pharmaceutical chemical drugs and compounds.

1.3 Aim

The aim of this research is to study the effect of electroporation on HeLa cell properties cultured on ECM protein coated surface.

1.4 Objectives

This study embarks on the following objectives:

a) To investigate the electroporation effect on HeLa cell cultured on Self Assembled Monolayer and micro fabricated ECM protein surface.
b) To analyse the growth, proliferation rate, and alignment of the HeLa cell cultured on the two different surfaces mentioned.
c) To analyse the cell attachment factor on 2 different type of protein (fibronectin and fetal bovine serum).

1.5 Scopes of study

In order to achieve the objectives of this research, the following will be the scopes of work identified:

a) To use the PDMS stamp for patterning protein.
b) To culture and grow HeLa cells on self-assembled surface
c) To culture and grow HeLa cells on the micro patterned surface
d) To expose the plated cells with microsecond pulse via high voltage pulse generator with 2 kV/cm voltage, 30 µs pulsed length and single number of pulse.

CHAPTER 2

LITERATURE REVIEW

2.1 Electroporation

Electroporation or electropermeabilization, is a phenomenon where cell membrane permeability is increased to ions and macromolecules by exposing the cell to short high voltage field pulses (Ivorra A. and Rubinsky B., 2006). It is also called a viable technique where, a short duration pulses are applied to temporarily open up the pores on the cell membrane. This is due to the field enhancement in the plasma membrane of cells that allow transporting or introducing of therapeutic materials including drugs, antibodies, genes (DNA) and RNA as shown on Figure 2.1 (Mark J. J. et al., 1999, Sundararajan R. et al., 2012a, Rodamporn S., 2012 and Chunlan J. et al., 2015) which otherwise are impermeable (R. Sundarajan et al., 2011). In the cell electroporation, the applied electric field intensity influences the cell viability (Min-Ji K., Taeyoon K. and Young-Ho C., 2011). In comparison with other methods of gene transfer, electroporation is a non-invasive and nonchemical method. This method does not change the biological structure and function of the target cell itself (Rodamporn S., 2012).

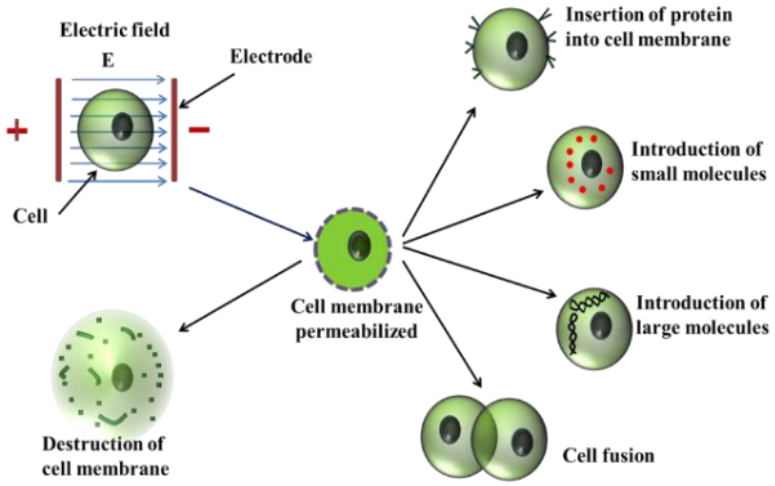

Figure 2.1: Different application of single cell electroporation (Santra T. S., Pen-cheng W. and Gang F., 2013)

Inducing the electrical pulses across cells can have a variation of results ranging from no effect to reversible and irreversible electroporation (Ivorra A. and Rubinsky B., 2006). Electroporation can lead to either reversible, which induced temporal pores in membranes and cell survive after removing the electric field, or irreversible, which induced pores in the membrane which are permanent that will cause the cell to die because of lysis (Vera Tizalt A. L. et al., 2013). Electroporation is a simple and easy technique that found to be effective for the intracellular delivery of molecules in living tissue, which led to variety of medical applications (Dev S. B. et al., 2000) in the treatment of tumours, genetics, immunology (Miklavcic D. and Towhidi L., 2010) and focal ablation of undesirable tissue. The most important parameters for the efficiency of this technique are the strength and duration of applied electrical pulses (Mengxi W. et al., 2013). Recently, there were many researchers who had proven the parameter of electroporation change depending on the high voltage discharge waveform, which is varied with respect to rise time, peak voltage and fall time. Moreover, the pores opened for about a few seconds or microseconds depending on the size of the pores and duration of time (Rodamporn S., 2012).

2.2 Electroporation (EP) system

In a complete experimental process of electroporation system, there are four main equipment required. There are inverted microscope (Ti-E series, Live Cell Instrument), stimulation magnetic chamber, square wave pulse generator (ECM® 830, BTX Harvard apparatus), and a system control that used a Methamorph® microscopy automation and image analyse software ver. 7.5.0.0. It is integrated with a computer work station. The efficiency of EP depends on the pulse amplitude, duration, repetition frequency, number of pulses and pulse shape (Miklavcic D., and Towhidi L., 2010, Stankevič V. et al., 2013). Nevertheless, various biological application required different parameter as shown in Table 2.1.

Table 2.1: Examples of electroporation parameters and its applications (Miklavcic D., and Towhidi L., 2010, Stankevič V. et al., 2013).

Pulses	Amplitude	Application
10-1000ns	Up to 10kV	▪ For affecting intracellular structures ▪ Processes and /or creating large number of small pores in the cell plasma membrane
1-10000µs	100 – 3000V	▪ Permeabilization of the cell plasma membrane (creation pores of variable size)
1-20ms	10 – 50V	▪ For affecting the transfer of large charged molecules (proteins, DNA, RNA)

2.3 Electroporation types

2.3.1 Reversible Electroporation (RE)

Electroporation is reversible which made temporal pores in membranes and cell survival after the removal of the electric field. Reversible electroporation is primarily

used for delivery of molecules into the cell. RE is often used to introduce substances into cells, such as dyes, drugs, protein and nucleic acids (Paula A. G., John H. R. and Rafael V. D. 2011, Sundararajan R. et al., 2014). The temporary opening of the cell membrane after the electroporation allows the cell to survive and this is called reversible EP.

2.3.2 Irreversible Electroporation (IRE)

Other than reversible, there is irreversible electroporation which make permanent pores in the cell membrane. This technique has shown great promise in the focal ablation of undesirable tissue (Paula A. G., John H. R. and Rafael V. D. 2011), sterilization of liquid media from microorganism (Ivorra A. and Rubinsky B., 2006), as a minimally invasive surgical procedure (Paula A. G., John H. R. and Rafael V. D. 2011) and neoplastic tissue ablation without scar formation or local bleeding. Under this circumstances, the electric field-induced pores in the membrane is permanent which causes the cell to die because of lysis. This happen when the magnitude of the induced transmembrane potential is above a critical value that the cell membrane is disrupted to the extent that the cell dies due to loss of homeostasis (Davalos R.V. et al., 2004, Paula A. G., John H. R. and Rafael V. D. 2011).

2.4 Electroporation applications

Application of electroporation is mostly established as a promising technique in several areas. It is mostly applied in two areas, namely biotechnology and medicine. There have been many emerging biotechnological electroporation-based applications. There are four general types of such applications which are electrotransformation, electroextraction, electroporation-based inactivation and biomass drying. Its utilization in medicine includes electrochemotherapy, gene therapy through electrotransfer, DNA vaccination, and tissue ablation.

2.4.1 Application in biotechnology

2.4.1.1 Electrotransformation

Although some microorganism can spontaneously transform by taking up foreign genes, expressing and replicating and passing them on upon division, the efficiency is nevertheless often low. Many approaches have been attempted. Since the mid-1980s, transformation based on electroporation which is called electrotransformation has succeeded because it is more efficient and applicable to a broader range of bacteria (Aune T. E. V. and Aachmann F. L., 2010). The applications of electrotransformation in the production of biomolecules are widely practised. Electro transformation is used for the synthesis of a foreign substance, including antigens, cytokines, enzymes, hormones, and toxins, in host organisms ranging from bacteria to microalgae and yeast. Transfer of gene into a simpler organism can simplify analysis of the properties and functions of the encoded protein, which may be concealed in the more complex original organism. If transgenic protein interacts with host genes or their expression, electrotransformation can also be used to study the genes and protein of host organism (Kotnik T. et al., 2015).

2.4.1.2 Inactivation of microorganisms

Inactivation of microorganism by electroporation has already been recognised since the 1960s and proved to be effective for increasing the shelf-life of liquid food. The use of electroporation for microbial inactivation is often called pulsed electric field (PEF) treatment. One of its applications is wastewater treatment which uses irreversible electroporation for bacterial decontamination of hospital wastewater, and also eradicates antibiotic-resistance strains. Consequently, this limits the spread of such bacteria into the environment. Nonthermal food pasteurization is a successful application of using electroporation as a mechanism of microbial inactivation in foods. This application strongly depends on several aspects: electric field strength, duration,

energy delivered, electric properties of the treated food, as well as microbial characteristics, including shape, size, cell wall structure, composition, and growth conditions (Mahnič-Kalamiza S., Vorobiev E. and Miklavcic D., 2014). This application of electroporation for microbial inactivation mostly aimed at food pasteurization than sterilization (Kotnik T. et al., 2015).

2.4.1.3 Electroextraction

Microorganism is being recognised as a potential source of various biomolecule for industry, pharmacy or medicine. A well-known technique to extract biomolecules includes mechanical forces or chemicals, which can be harmful to the structure and/or integrity of extracted biomolecules. Furthermore, after total microorganism disintegration, purification or targeted biomolecules from cellular debris is needed. This is often expensive as it requires additional steps in the process. In difference from the extraction by electroporation, electroextraction it is a fast, chemical-free, energy saving technique, allows rapid microorganism disintegration resulting in debris to be evaded and easily up-scalable (Kotnik T. et al., 2015).

2.4.1.4 Biomass drying

Electroporation is used in electroporative biomass drying for facilitating water released from tissues. It also speed up the drying process, allowing heating to be condensed or avoided, and also reducing the energy requirement. The Electroporation-based treatment of green biomass can be done in electrified press. In this treatment, mechanical force is applied before and during pulse application, forming electric contact to the electrodes through extracted juice without the need to add water. When drying the biomass in an oven, the electroporated material dries 2-3 folds faster that non-porated materials. This is not only because of reduced water content after pressing but also because of improved diffusion of vapour as a result of cell disintegration (Kotnik T. et al., 2015).

2.4.2 Application in medicine

2.4.2.1 Electrochemotherapy

Electrochemotherapy is an electroporation-based cancer treatment methods are currently undergoing thorough investigation in the field of drug delivery and gene therapy. Which begins in the late of 1980's has evolved into a clinically tested treatment for cutaneous and subcutaneous tumour nodules (G. Sersa et al., 2009). The termed "Electrochemotherapy" comes from the combination of electroporation and chemotherapy or in short "ECT".

Electrochemotherapy is an application of cell membrane permeabilizing by electric pulses and has been used as a local treatment. The principal mechanism of electrochemotherapy is the electroporation inducement of tumours, a process that increases the drug effectiveness by enabling the drug to influence the intercellular targets (Sersa G. et al., 2009). Application of electric pulses to the tumors can be performed either by plate electrodes that are placed on the skin above the tumors or by needle electrodes that are inserted into them (Marty M. et al., 2006). The advantages of electrochemotherapy are efficient, safe inexpensive once-only treatment that can be offered to the cancer patients with tumors of a variety of different histology (Anita G., Mir L. and Gehl J., 2003, Sundararajan R., 2011a).

2.4.2.2 Gene electrotransfer for gene therapy and DNA vaccination

The use of electric pulses is very general for the transfection of bacterial and eukaryotic cells in vitro. The initial restraint of electroporation method is, a low cell survival, could be overcome by the use of suitable electric pulse. This method is then transferred to in vivo and called DNA electrotransfer or electrogene therapy. DNA electrotransfer has been used well since 1988 and become a real alternative to the viral methods for in vivo gene transfer.

The first revolutionary demonstration that DNA could be introduced into living cells by using electric pulses was published by E Naumann in 1982. Since 1986, DNA electrotransfer is the most well-known way to transfer bacterial cells and one of the good preferences for the in vitro transfection of eukaryotic cells as well (Andre F. and Mir L. M., 2004). Gene electrotransfer is the most effective and safest non-viral gene transfer technique and particular electrical parameters have been developed for some target tissues (Christophe Y. C. et al., 2014).

2.5 In Vitro methods

In vitro method is the most popular technique that has been used by the researchers at earlier phase of the experimental such as electroporation method that used in this study. In vitro methods is the technique of performing an experimental in a controlled environment outside of living organism (Straughan D. W. et al., 1997). For this study, it is introduced the cellular level interactions with the pulsed electric field exposed under control environments.

The advantage of this method is that some of the exposure circumstances can be easily controlled. An example of this is the exposure of field intensity. The disadvantages of in vitro testing are the tissue and cells are isolated from the complete complex system of the body. Any effect that observed in vitro needs to be considered back to the whole body system scenario (Adon M. N., 2015).

2.6 HeLa cell

HeLa cell line is a cancer cell type, which is an immortal cell line that is commonly used in scientific research. It was derived from human cervical cancer taken from Henrietta Lacks, a patient who died of cancer in 1951. The name of HeLa is based on the first 2 letters of her name. It is the oldest and most generally used human cell line. The main advantage of this cell is that they do not die after a specific growth cycle and

can be divided to an unlimited number of times as long as basic cell survival conditions are met. Therefore, HeLa cell was used as the primary cell type in this study.

2.7 Cell attachment

Cell attachment is an interaction or contact within cells precisely with other cells through various cellular junctions. They also bind with the extracellular matrix, which is a complex network of secreted protein and carbohydrates that playing a major role in communication between the cell and its surroundings.

Cell attachment assays measure adhesion between cells, between cells and surface and between the cell and extracellular matrix proteins. Besides, the biological process that involves in adhesion is complex. It is involving molecular interactions, ligand binding and intercellular signalling affecting different cell processes such as growth, differentiation, and metastases. Cell adhesion assays have been applied in many areas such as disease research (Lodish H., Berk A., Zipursky S. L., et al., 2000).

2.8 Cell adhesion

Cells are rarely found in isolation. Instead, they usually stick to the other cells or non – cellular components of their surroundings. Fundamentally, cell adhesion mostly involves protein molecule at the surface of cells. Thus, the study of cell adhesion involves cell adhesion proteins and the molecules that they bind to as shown in Figure 2.6 (Abdul Jamil M. M.., 2007).

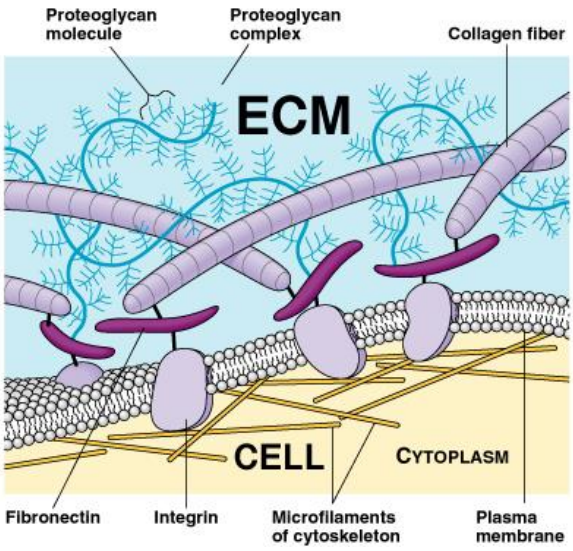

Figure 2.2: Diagram of cell adhesion with ECM (J. Imbeau and S. Thouin., 1999)

Protein and enzymes are really interesting for nanobiotechnology and nano electronics due to their ability of self-assemble. This opens the possibility of nanoscale surface patterning. Furthermore, due to their great potential in controlling highly specific reactions, protein and enzymes can be optimized towards the different technologies target (R. Rinaldi et al., 2004). The interaction of cells with their environment influences their internal functions and cellular behaviour (James H-C. W. & Bin L., 2010). The extracellular matrix (ECM) is one of the important components that influences cell interaction and function during development, maintenance, and regeneration. Integrin's bind to a specific ECM protein, in particular to amino acid sequences connecting the adhesive regions (Yoshihiro I., 1999).

The ability to generate a micro pattern of protein and cells on a substrate is important for biosensor technology (Thomas C. A. et al., 1972), tissue engineering and also for fundamental studies of cell biology (Mrksich M. and Whitesides G. M., 1996a). In tissue engineering, the cells are required to be in specific locations to create organized structures. This is because the patterning technique can control both the size and shape of the cells that are attached to the surface of the substrate in order to understand the influence of the cell materials interface on the behaviour of the cells (Singhvi R. et al., 1994, Chen C. S. et al., 1997, Lopez G. P. et al., 1993, Mrksich M. et al., 1996, Ravi S. K. et al., 1999).

2.9 Cell adhesion protein

Cell adhesion is usually transmembrane receptors. The transmembrane cell adhesion protein can extend across the cell surface membrane and characteristically has domain that extends into both extracellular and also intracellular space as shown on figure 2.7(Abdul Jamil M. M., 2007, Uhlen et al., 2015).

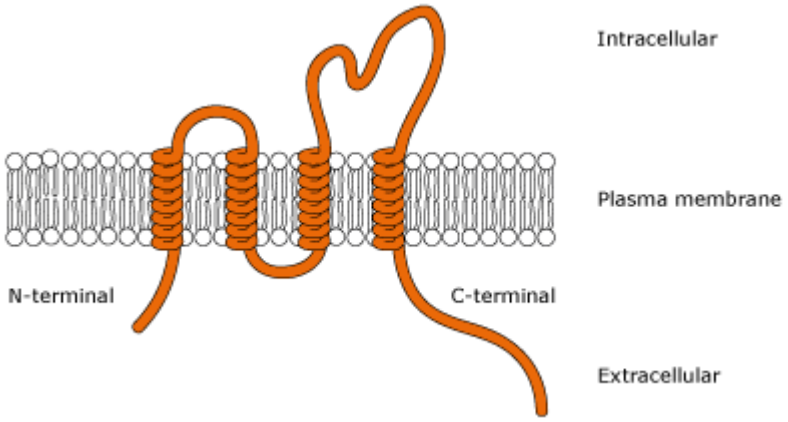

Figure 2.3: Illustration of transmembrane receptors

There are two types of extracellular domain of a cell adhesion protein that can bind to other molecules which can be either cell to cell adhesion or cell to extracellular matrix adhesion (Sunami H. et al., 2006, Lodish H., et al., 2000, Alberts B. et al., 2002).

2.10 Extracellular matrix (ECM)

Extracellular Matrix or (ECM) is a non-cellular component that exist within all tissues and organs. It is collection of extracellular molecules secreted by the cells that provides structural and biochemical support to the surrounding cells. It can provide an essential physical scaffolding for the cellular constituents. ECM can initiates crucial biochemical and biomechanical cues that required for the tissue morphogenesis, differentiation and homeostasis.

The common function of ECM is cell adhesion, cell to cell communication and differentiation. ECM in animal cells consist of basement membrane to interstitial matrix (contain gel of polysaccharides and fibrous protein). ECM can serve many function such as providing support, separating tissues from one another and also regulating intercellular communications. The formation of ECM is important for the process of growth, wound healing and fibrosis (Alberts B. et al., 2002, Christian F. et al., 2010). The ECM can be categorized as two major classes of biomolecules that is glycosaminoglycans (GAGs), which normally linked to protein forming the proteoglycans, and fibrous proteins which contain of elastin, collagen, fibronectin, and laminin. ECM is made by these components that are secreted and assembled into the organized meshwork.

The ECM is not just for connecting cells together to form tissues. It is also a substrate upon which cell migration is guided during the process of wound healing and during embryonic development. In addition, the ECM is in charge for the transmitting of environmental signals to the surfaces of individual cells (Michael W. K., 2016).

2.11 Fibronectin

Fibronectin (FN) has been used as a mediator to form a connection between cells and the ECM. It plays a main role in cell adhesion, migration, growth, and differentiation, and also important for developments such as wound healing and embryonic development. Besides, Fibronectin is a dimeric glycoprotein, comprised of dimmers, each of it approximately 250,000 molecular weight. Fibronectin is one of the ECM proteins that have been used to assist in the wound healing process by facilitating cell movement to the affected area and binding to platelets during blood clotting.

2.12 Fetal bovine serum

Fetal Bovine Serum (FBS) is a common supplement that is used for in vitro cell culture media in order to keep the cell alive. Fetal Bovine Serum is harvested from the bovine fetus taken from a pregnant cow during slaughter. FBS has a very low level of antibodies and containing supplementary growth factors, allowing for versatility in many different cell culture applications. The rich variation of proteins in fetal bovine serum preserves cultured cells in a medium in which they can survive, grow, and divide. (Van der valk J. et al., 2004).

2.13 Cell guidance

Cell guidance is used to control the attachment of cells on substrate for biological studies by controlling the shapes and position of attached cells to the planar substrate. (Mrksich M. et al., 1997). Guidance of cells is important for many applications such as tissue engineering (Johansson F. et al., 2010). Controlling the migration of adherent cells is very useful for studying cellular mechanics and response to external stimulation (Xiao J. L. et al., 2013). Most studies have been performed on micrometre sized structure, such as grooves and ridges, due to technical limitations (Johansson F. et al., 2010). Track of adhesiveness is believed to be involved in guiding morphogenetic cell migrations (Clark P. et al., 1992) such as surface properties of ECM. It plays a vital role in cellular activities in adhesion spreading, migration, proliferation and differentiation (Zhu B. et al., 2004).

2.14 Cell proliferation

Cell proliferation is the process of increasing number of cell. It is define by the balanced between cell divisions and cell loss through cell death or differentiation. The proliferation of mammalian cells is firmly controlled by multiple environmental

influences, primarily adhesion to the extracellular matrix, cell-cell adhesion and soluble factors of these environmental cues, soluble growth factors and integrin-mediated adhesion (Martin A. S. and Richard K. A., 2001).

2.15 Wound healing

Wound healing is a complex and active process of replacing devitalized and missing cellular structures and tissue layers. The human adult wound healing process can be divided into 3 or 4 distinct phases. Within 4-phases concept, there is haemostasis, inflammatory, proliferation, and remodelling phase. In the 3-phases approach, the haemostasis phase is contained within the inflammatory phase (Michael. M. and Adam J. C., 2015). Wound healing is essential for maintaining the integrity of multicellular organisms (Gang Yang et al., 2008). Wounds are physical trauma due to the tissue is torn, cut or disrupted and resulted in blood and fluid loss and increase the risk of bacterial invasion and infection. Wounds triggers the body responses, and certain mechanism design to restore tissue integrity, structure, and function. This is called a protective responses that induced by the various exogenous and endogenous stimuli in order to eliminate the damage that caused by the induced injury. The inflammatory phase is response which triggers a healing process that organises of necrotic cells and tissue and generates new tissue. During reparative phase, injured tissue is replaced by parenchymal cells or fibroblast scar tissue. Normal function and structure of tissue can be restored if the tissue is able to regenerate and the damage is small. Extensive injury to tissue that is unable to regenerate would result in scarring. Infections that cannot be removed, resulting in abscesses (Michael M. and Adam J. C., 2015, Stroncek J. D. and Reichert W. M., 2008).

2.16 Self-assembled monolayer

Self-assembled monolayers (SAMs) also known as a spontaneous organization are typically formed from the exposure of a surface to molecules with chemical groups

that possess strong affinities for the substrate or a material patterned on it. How well these assemblies order is a function of the nature of the chemical interaction between substrate and adsorbate, as well as the type and strengths of intermolecular interactions between the adsorbates that are necessary to hold the assembly together. Molecules ‘‘binding to’’ surfaces are either described in terms of physisorption, or in chemisorption (Rahul B. and Anil M., 2010, Rachel K. S., Penelope A. L. and Paul S. W., 2004, Abraham U., 1996).

2.17 Micro contact printing

Micro contact printing (μCP) has been developed around 20 years ago and it is an outstanding surface patterning technique. Surface science engineer communities like engineers and biologist have been promoting an attention on micro contact printing (Maksud M. I., Yusof M. S., and Abdul Jamil M. M., 2013). Micro contact printing is quite a useful technique for the patterning of extracellular matrix (ECM) by working as an adhesion molecule for cell attachment (Nobuyuki T. et al., 2013). Its application covers a wide range of applications including microelectronic, surface chemistry, cell biology (Xia Y. and Whitesides G. M., 1998) biotechnology (Dong Q., Xia Y. and Whitesides G. M., 2010), tissue engineering, cell cultures, bio-assays, biosensor, and many others (Azioune A. et al., 2010).

μCP significantly had a large impact on study of control cell growth (Quist A. P., Pavlovic E. and Oscarsson S., 2005) and cell guidance (Clark P. et al., 1992). Polydimethylsiloxane (PDMS) is the material that is mostly used to make the stamp, since it can be moulded by using a master template (Quist A. P., Pavlovic E. and Oscarsson S., 2005). This is because PDMS is transparent material, which is significant for optical applications and process control by the eye and by microscope observation (Maksud M. I., Yusof M. S., and Abdul Jamil M. M., 2013). It can be produced easily by thermally curing the cheap and commercially available pre-polymer for a few hours (Tobias K. and Bart. J. R., 2010, Maksud M. I., Yusof M. S., and Abdul Jamil M. M., 2013).

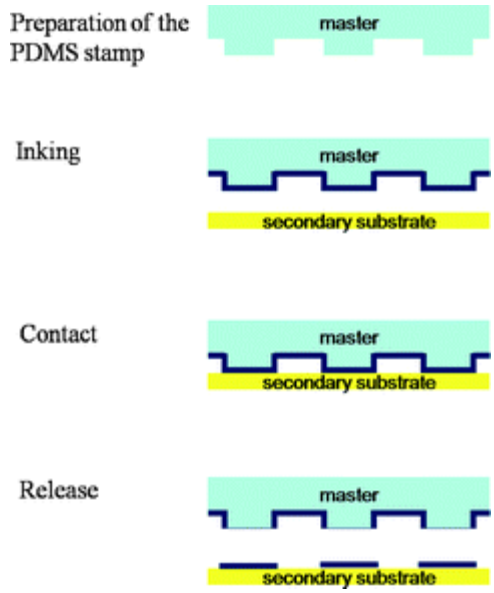

Figure 2.4: Micro contact printing process (Akbulut O., Yu A. and Stellaci F., 2010)

µCP is a soft lithography that used the release patterns from the master of polydimethylsiloxane (PDMS) stamp to form the patterns of self-assembled monolayers (SAMs) ink on the surface of a substrate through conformal contact (Maksud M. I., Yusof M. S., and Abdul Jamil M. M., 2013). It is used to study the role of spatial signalling in cell biology by controlling the molecular structure of a surface in contact with cells (Chen C. S. et al., 1997). The advantage of micro contact printing is, once a mould is available to produce the stamps, no special equipment and special chemistry are required to produce patterns (Thery M. and Piel M., 2009, Ostuni E. et al., 2009). It can adapt to various substrates (such as glass, plastics, hydrogels, and elastomers), molecules and cell types, in two and three dimensions (Azioune A. et al., 1997). It can also be used with a variety of inks (Xia Y. and Whitesides G. M., 1998). There are few types of patterning, such as patterning proteins, patterning cells and patterning DNA that are used in micro contact printing. First, for patterning protein numerous proteins have been proven to be suitable for inks and applied to different substrate using the micro contact printing technique. Such as, polylysine, immunoglabin, antibody and different enzymes have been successfully placed onto the surfaces including glass, polystyrene and hydrophobic silicon. This method helps in the development of biosensor, cell biology research and tissue engineering. While, for the patterning cells is used in order to have an advance understanding of how cell interacts with substrate. This technique helps in improving the study of patterning the cell that is not possible with traditional cell culture techniques. Lastly, for the

patterning DNA, it has been done using micro contact printing technique in nanometre scale. The study of patterning DNA has been taken seriously for the miniaturized tools for pharmaceutical and diagnostic applications as well as emerging area of DNA-based nanoassembly of electronic devices. The patterning DNA on surface have enabled the rapid development of biosensor for the DNA analysis (Yin H. B. et al., 2004).

2.18 Combination of μCP and EP as an alternative

Electroporation is a method that can increase the cell proliferation and cell attachment by inducing an electrical field to the cell by using reversible electroporation method. This happens due to opening pores of the cell membrane and allows substances around it to be absorbed into the cell. It has been used in many applications such as introduction of therapeutic materials including drugs, antibiotic, and gene (DNA) and RNA (Mark J. J. et al., 1999). While micro contact printing is a method that can control the cell shape and size, it has resulted in contribution towards cell guidance and cell alignment that attached to the substrate. Based on the literature review these two methods can be combined in the in-vitro environment to observe the effect and cell response.

The combination of this two method potentially will enable a new method of wound healing process since there is still minor research reported on the combination of these two methods. Both EP and μCP were found to be related to wound healing process. The parameter of pulsed electric fields that induce to the cell and the size of pattern for the μCP were studied in order to get a result for the cell guidance. The EP parameter was set up to produce a reversible electroporation and the size of pattern was selected by choosing pattern that is suitable with the size of cell.

In the next chapter (**Chapter 3**), the details setup of electroporation methods, micro contact printing technique and cell culture protocols will be demonstrated to give a clear view on the project methodology.

CHAPTER 3

ELECTROPORATION METHODS, MICROCONTACT PRINTING TECHNIQUE AND CELL CULTURE PROTOCOLS

3.1 Introduction

This chapter present about in vitro technique to evaluate HeLa cells interaction that was exposed to the electric field under controlled environments outside living organism. The research also analysed the interaction of HeLa cell on micro patterned surface. Electroporation is believed to have the capability to control cell functions using electric field to create pores through cell membrane. This has led electroporation to be used in a variety of medical applications. Micropatterned has been able to control cell behaviour by adhesive surfaces. Thus, in this chapter the flowchart, the details setup of electroporation methods, micro contact printing technique and cell culture protocols is demonstrated in order to give a clear view on the project methodology of this study.

3.2 Flowchart of study

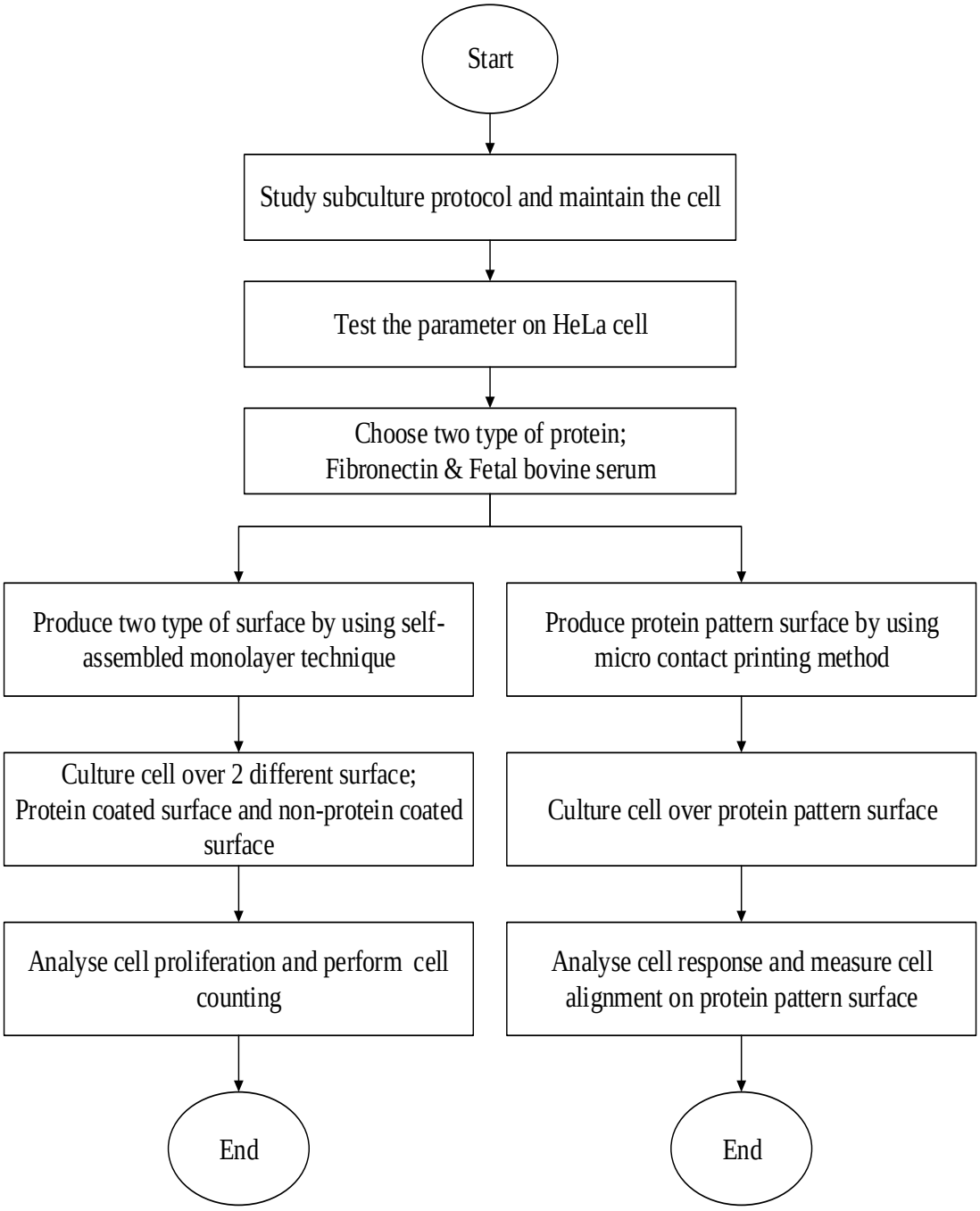

Figure 3.1: Flowchart of study

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