INVESTIGATION ON HELA CELLS BEHAVIOUR
INDUCED WITH PULSE ELECTRIC FIELD FOR
WOUND HEALING APPLICATION

MOHAMED AHMED MILAD ZALTUM

UNIVERSITI TUN HUSSEIN ONN MALAYSIA
INVESTIGATION ON HELA CELLS BEHAVIOUR INDUCED WITH PULSE ELECTRIC FIELD FOR WOUND HEALING APPLICATION

MOHAMED AHMED MILAD ZALTUM

A thesis submitted in fulfillment of the requirement for the award of the Degree of Doctor of Philosophy in Electrical Engineering

Faculty of Electrical and Electronic Engineering
Universiti Tun Hussein Onn Malaysia

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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.

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ABSTRACT

This study focuses on the investigation of pulsed electric field (PEF) exposure effect on HeLa cells (cervical cancer cells) for in-vitro experiments. The study focused mainly on real time experimental setup for cell morphological properties imaging. In the experimental setup, a modified EC magnetic chamber with incubator system is used to maintain the real time in-vitro environment for exposing the HeLa cells to high electric field. A Nikon inverted microscope (Ti-series) with Metamorph® time lapse application is utilized for image capturing and video recording. The first investigation is to look at the proliferation rate of HeLa cells within 72 hours inside the modified chamber. This first investigation is utmost important to see if there is any effect from the PEF exposure. From this, it was found that the HeLa cell growth rate increased up to 50% faster when applied Electroporation (EP) in comparison to the cell without EP treatment. The investigation was continued to look at the best PEF parameter that assisted in the growth rate of HeLa cells. These investigations motivate the need of finding the best parameters for PEF exposure that are suitable for HeLa cell reversible condition. This study continues to look at the relation between amplitude and duration of PEF effect on the HeLa cell growth rate. The cells were subjected to single pulse, constant field strength of 1kV/cm and pulse durations ranging from 30μs to 600μs. It was found that at 100μs pulse duration the HeLa cell growth rate increased dramatically and achieving confluency faster in comparison to the cells exposed with other pulse durations. After obtaining the best parameter for HeLa cell (1kV/cm, 100μs, & single pulse) the potential application of the EP technique for wound healing was explored. The result of the exposed cells to PEF revealed a five times faster healing rate than control group. Additionally, we have used Microcontact printing (MCP) technique for cell guidance and assembling. The results indicate that the cells aligned and elongated more on fibronectin pattern substrate under PEF than without PEF. Thus, PEF usage on biological cells would enable a novel method for assisting drug free wound repair systems and many other potential biomedical engineering applications.
Kajian ini memberi tumpuan kepada penyiasatan kesan pendedahan denyutan medan elektrik (PEF) pada sel HeLa (kanser pangkal rahim) dalam ujian eksperimen secara in-vitro. Kajian ini memberi tumpuan terutamanya kepada persediaan eksperimen pada masa sebenar untuk pengimejan sifat morfologi sel. Dalam persediaan eksperimen, kami menggunakan ruang magnet EC yang diubahsuai dengan sistem inkubator untuk mengekalkan persekitaran in-vitro pada masa yang sebenar untuk mendedahkan sel HeLa dengan medan elektrik yang tinggi. Mikroskop Nikon inverted (Ti-siri) dengan Aplikasi time lapse Metamorph® digunakan untuk memantau, tangkapan imej dan rakaman video. Siasatan pertama adalah untuk melihat kadar percambahan sel HeLa dalam masa 72 jam di dalam ruang medan EC yang diubah suai. Kajian pertama ini adalah penting untuk melihat jika terdapat sebarang kesan masa nyata daripada pendedahan PEF itu. Hasil didapati bahawa kadar pertumbuhan sel HeLa meningkat sehingga 50% lebih cepat apabila menggunakan elektroporasi (EP) berbanding dengan sel tanpa rawatan EP. Siasatan diteruskan dengan melihat parameter optimum PEF dalam membantu kadar pertumbuhan sel HeLa. Penemuan ini mendorong kami untuk melihat parameter optimum untuk pendedahan PEF yang sesuai untuk sel HeLa keadaan berbalik. Kajian diteruskan dengan melihat hubungan antara amplitud dan tempoh kesan PEF pada kadar pertumbuhan sel HeLa. Sel tertakluk kepada denyut tunggal, pada kekuatan medan berterusan 1kV/cm, pada jangka masa nadi antara 30μs hingga 600μs. Ia juga mendapati bahawa pada kadar tempoh denyutan 100μs pertumbuhan sel HeLa meningkat secara mendedak dan mencapai kadar pertumbuhan lebih cepat berbanding dengan sel yang terdedah dengan jangka masa nadi yang lain. Selepas mendapat parameter optimum untuk sel HeLa (1kV/cm, 100μs, & denyut tunggal) potensi aplikasi tekik EP untuk penyembuhan luka telah dikaji. Hasil daripada pendedahan sel kepada PEF menunjukan lima kali lebih cepat dalam kadar penyembuhan daripada kumpulan kawalan. Selain itu, kami telah menggunakan teknik Microcontact Printing (µCP), untuk memberi panduan pada sel dan juga pengumpulan. Keputusan menunjukkan bahawa sel sejajar dan memanjang mengikut corak fibronectin dengan PEF berbanding tanpa PEF. Oleh itu, penggunaan PEF pada luka sel biologi akan mewujudkan kaedah baru untuk membantu sistem perbaikan luka tanpa bantuan ubat dan lain-lain yang berpotensi dalam aplikasi kejuruteraan bioperubatan.
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<tr>
<td>ECM</td>
<td>Extra Cellular Matrix</td>
</tr>
<tr>
<td>EP</td>
<td>Electroporation</td>
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<tr>
<td>PDMS</td>
<td>Polydimethylsiloxane</td>
</tr>
<tr>
<td>PEF</td>
<td>Pulse Electric Field</td>
</tr>
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<td>HT29</td>
<td>Colon Cell line</td>
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<td>HeLa</td>
<td>Cervical Cancer Cell</td>
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<tr>
<td>J3T</td>
<td>Brain Tumors Cells</td>
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<td>CHO-K1</td>
<td>Chinese Hamster Ovary</td>
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<td>ECT</td>
<td>Electrochemotherapy</td>
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<td>EGT</td>
<td>Electrogenetransfection</td>
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<tr>
<td>EF</td>
<td>Electofusion</td>
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<td>EI</td>
<td>Electroinsertion</td>
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<tr>
<td>TDD</td>
<td>Transdermal Drug Delivery</td>
</tr>
<tr>
<td>MCP</td>
<td>Micro-Contact Printing</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
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<tr>
<td>NEP</td>
<td>Non-Electroporation</td>
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LIST OF PUBLICATIONS, RESEARCH GRANT AWARD

The followings are the list of publications associated with this thesis:

Journals:


Book chapter:


Conference proceedings:


9. **Mohamed A. Milad Zaltum** & Muhammad Mahadi Abdul Jamil. Optimization of Pulse Duration Parameter for HeLa cells Growth Rate. International Conference on Biomedical Engineering (ICoBE 2017), School of Mechatronic Engineering, Universiti Malaysia Perlis (UniMAP). *(In review).*
Research grant award:

Grant Type: Fundamental Research Grant Scheme award (FRGS) phase 2/2014, Vot 1488, Title: **Fundamental Study On Hela Cells Morphological Properties Induced Via Microsecond Pulse**. Funded by: Ministry of Education Malaysia, 16 November 2014.
CHAPTER 1

INTRODUCTION

The focus of this thesis will be on the use of electroporation system to investigate the HeLa cell morphological properties. Recent developments in electroporation have enabled a broad range of biological applications. For example, the use of electroporation systems in biotechnology and medicine has led to new methods of cancer treatment, gene therapy and drug delivery. Electroporation is an alternative technique that enables the delivery of foreign materials into the cell by applying an electric field applied across a cell opens pores in the cell membrane. The effects of electric field on human body and cells that are recently discovered are discussed this chapter.

1.1 Basic information of electroporation

Living cells exhibits various electrical properties that make them to react and create electric field and current (Hondroulis et al., 2013). Therefore, controlling the electrical characteristic of the cell can provide a significant direct healing choice for wound repair application and cancer treatment (Hondroulis et al., 2013). A technique that uses high voltage electrical pulse to tissue in-vivo or cells in-vitro is knows as Electroporation. This technique was found to improve the cell’s uptake of molecules by producing transient pores in the cell membrane (Titushkin et al., 2009).

The effect of electroporation is not only extended to opening pores in the cell membrane, but it can also control the variations in the cytoskeletal restructuring. Thus, it has a noticeable impact on the cell adhesion and migration (Titushkin et al., 2009). Though, the physiology of cells can be altered with the help of electric field by involving intracellular signalling pathology yet the entire process is not fully
understood and need further research. Mamman and Abdul Jamil (2015) have stated that the electric field can significantly affect the attachment speed and spreading characteristic of colon cell line (HT29) by exposing them to an electric field of 0.6kV/cm for 500µs duration (Mamman et al., 2015).

*In-vitro* methods for human cell culturing are adopted by many recent researchers for applying electroporation. Melanoma cells (Daud *et al.*, 2008), J3T (brain tumours) cells (Andre & Mir, 2004), and HeLa (cervical cancer) cells (Nazib *et al.*, 2013) are the cell types used in electroporation by different researchers. Different types of cells can be classified and used based on their structure, shape and content. However, different types of cells exhibits different characteristics towards electric field intensities and duration, hence the selection of cells is very important in the process of electroporation.

In recent years, Yong Hung and Boris Rubinsky (2003) have designed a new micro fabricated electroporation chip for single cell membrane permeabilization (Yong *et al.*, 2003). They incorporate a live biological cell in the electrical circuit of a micro electroporation chip. Moreover, they investigated the fundamental biophysics of membrane permeabilization on a single cell level. It can control introduction of macromolecules into individual cells. The experimental results shows that the chip has a good ability to manipulate and induce electroporation in specific cells.

Furthermore, Rivera *et al.*, (2004) have designed a fluidic microchip to inject therapeutic molecules in the whole targeted tissue (Rivera *et al.*, 2004). The great advantage of this device is that it is a stand-alone device. The device uses gold electrodes and leads are passivized with silicon oxide. A stand-alone device is 500µm square sections, so that it is small enough to be inserted deep into a target tissue. It can apply high voltage electric impulse into therapeutic molecules, genes or drugs which are injected into targeted tissue. Moreover, the device is designed to allow electro transfection *in-vivo* because of its invasiveness.

The electroporation microsystem has been developed and combined with a logic circuit for gene transfection. Min *et al.*, (2005) have designed an electroporation microchip device for gene transfection and system optimization (Min *et al.*, 2005). The device combines micro fabrication techniques, logic circuit and electrophoresis design to create a multi-function gene transfection device. This device can be used in wide areas of medical science research applications. In their study, they subjected 104 NIH 3T3 cells to an electroporation process with a 50µm electrode gap, 6 volts and two
pulses applied. They used a fluorescence microscope to observe the experimental results. They have reported the efficiency of gene transfection with an electric field becomes higher than without electric field. The delivery rate was increased to 35.89% when putting GFP gene into NIH 3T3 cells. This device has been applied in cancer research, protein transfection, and drug delivery.

Many studies have been conducted in order to explore the mechanism of electroporation and its applications to cell fusion (Nazib et al., 2013), drug delivery (Prausnitz et al., 2004), gene therapy (Heller & Heller, 2006). From literature it can be observed that cells exhibits different kind of characteristics to pulsed electric field with different intensity and duration such as alteration in morphology of the cells. This leads to the suggestion of different applications of electroporation.

The implanted electrodes in cuvette are polished to achieve sterility. For real time visualization experimental setup cuvette alone cannot be used. Therefore, a customized setup is required to allow high resolution visualization of electroporation. Once designed with high resolution microscope, it can provide real time information of electric field introduction to cells. This customized setup also allows the researchers to investigate its effects on cell behaviour and introduction of foreign materials in cells.

1.2 Problem statement

Although, many research studies focus on pulse electric field effect, the main phenomenon of the effects of external pulse electric field on cells is still in process of discovery.

Different applications of electroporation require different EP parameters for different cells type. An intense external electric field could damage the cell membrane and lead to cell lysis. Therefore, examining of EP parameter for different applications is very significant and extended experimental knowledge with theoretical models is needed.

Normal electroporation setup in biology uses cuvettes which do not support real time observation. Therefore to observe the morphological characteristics of cells, an experimental system is required which uses live imaging for observation.

Even though electric field can alter the cell physiology by interacting with the cell signalling pathways, only little is known about the whole process. Similarly, there
is evidence that electric field can considerably influence adhesion and spreading properties of many cells. Since cell adhesion and migration are strongly associated, there is potential for investigating electric field in cell migration for drug free wound healing treatment.

The alignments of cells play a key role in wound healing application. Many researchers have investigated cell guidance and alignment via micro-contact printing with different protein. However, the inducement processes of micro-patterned in combination with electric field excitation towards cell guidance have not been investigated.

1.3 Objective of research

The main aim of this research is to observe the effects of PEF on growth rate of HeLa cells for wound healing application. To achieve this, the research objectives are divided into four parts:

1- To develop an experimental system setup that introduces electric field for electroporation of cells so that the process can be observed and improved in real time.

2- To optimize the best electroporation parameters for growth rate of HeLa cells in the experimental work.

3- To investigate the electroporation process on HeLa cells for applications in wound closure based on optimized electroporation parameters.

4- To investigate the HeLa cells interactions with micro-patterned surface assisted by pulse electric field excitation for cell guidance.

1.4 Scope of research

The following scope of research has been identified to achieve the objectives of this research:

1. Achieving the process of HeLa cell subculture in laboratory using the required equipment for experimentation process (Plating, Growing and Splitting of cells).
2. Acquisition of custom made of 1mm gap electrode chamber (EC Chamlide Magnetic Chamber).
3. Investigation of pulse duration exposure rate for electroporation process to get the best parameter for growth rate of HeLa cells.
4. Time-lapse live imaging for Investigation of drug free wound closure of HeLa in the presence of pulse electric field.
5. Printing of protein of various patterns on glass coverslip substrates and subsequent plating of the cells on the patterned surface for cell alignment under pulse electric field.

1.5 Thesis outline

The thesis is divided into eight chapters. Following are the brief content of each chapter:

Chapter 1: The structure of this thesis reflects and flow of information identified in overview of the research is focused in this chapter.

Chapter 2: An overview of the cell and electroporation application methods are presented in this chapter. The cell structure, electroporation and the parameters that affect their performance are also discussed.

Chapter 3: This chapter demonstrates HeLa cells culture protocol and the development process of the experimental setup for electroporation of HeLa cells.

Chapter 4: This chapter investigates the pulse electric field exposure effect on growth rate of HeLa cell reversible condition.

Chapter 5: The selection of best parameters of pulse electric field for growth rate of HeLa cells are shown in this chapter.

Chapter 6: This chapter gives the analysis of the wound closure process assisted by pulse electric field.

Chapter 7: In this chapter, the assembling and guidance of HeLa cells on micro patterned surface coated fibronectin with and without pulse electric field are investigated.

Chapter 8: The last chapter will conclude the overall findings and provide recommendation for future work.
1.6 Thesis contribution

In this research, the influence of pulse electric field on the cellular behaviour such as proliferation, migration and cell guidance of HeLa cell have been investigated. The following points demonstrate the main contributions of this research:

1. In the experimental setup, a custom made EC magnetic chamber is used to ensure that the electroporation process is performed under fully controlled environment. It maintains the real time in-vitro experiments for exposing HeLa cells to high electric field. It also allows the investigation of multicellular behaviour of HeLa cells during PEF exposure for qualitative and quantitative analysis.

2. Pulse Electric Field parameter is investigated to examine and to get the best value for PEF parameters (pulse strength 1kV/cm, pulse duration 100µs & single pulse) towards faster growth rate of HeLa cells. This can be used in clinical applications.

3. This research applies pulse electric field along with micro-contact printing technique on HeLa cells which facilitates cellular processes like migration, cell guidance and cell alignment. These cellular processes are vital for wound healing process, tissue regeneration and cancer treatment.
CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

This chapter presents the background and literature review of the state of the art work in the field of electroporation. Additionally, biological cells are discussed in this chapter providing more detail of the type of cells used in this research.

2.2 Introduction of living cells

Cells are the structural and functional unit of all living organisms and are called the “building blocks of life” (Mariana, 2007). They are divided into two types. Bacteria cells are prokaryotic; all other cells are eukaryotic of which there are four kinds: animal, plant, fungi and Protista. Prokaryotic and eukaryotic are compared in table 2.1. Prokaryotic cells (shown in figure 2.1) are smaller than eukaryotic cells (shown in figure 2.2). Prokaryotic cells have no nucleus and a typical cell size of less than 5μm. Typical cell mass is around 1 nano gram. This research used HeLa cell which is described in detail in section 2.2.1.
Figure 2.1: Prokaryotic cell (Source: http://www.shmoop.com/biology-cells/prokaryotic-cells.html)

Figure 2.2: Eukaryotic cell (Source: https://openoregonstate.pressbooks.pub/microbiology/chapter/introduction-to-cell-structure)
Eukaryotic cells typically contain additional components. Lysosomes are used to break down unwanted chemicals, toxins, and organelles. The cell membrane is a very flexible and thin layer surrounding the cells. The cell wall is a tough and thick layer outside the cell membrane. The cell wall is used to give a physical rigidity and allows chemical and cellular material to pass through the cells. These cells are found in plants, animals and fungi. The Undulipodium is a flexible tail whose function is to provide motility.

Table 2.1: Summary of the prokaryotic and eukaryotic cells type (Mariana, 2007)

<table>
<thead>
<tr>
<th>Prokaryotic cells</th>
<th>Eukaryotic cells</th>
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<tr>
<td>Size of cells $&lt; 5\mu m$</td>
<td>Size of cells $&gt; 10\mu m$</td>
</tr>
<tr>
<td>Exist in unicellular form</td>
<td>Exist in multicellular form</td>
</tr>
<tr>
<td>Do not contain nucleus or any membrane-bound organelles</td>
<td>Have nucleus and other membrane-bound organelles</td>
</tr>
<tr>
<td>DNA is round in shape which do not contain proteins</td>
<td>DNA is linear and associated with proteins to form chromatin</td>
</tr>
<tr>
<td>Do not contain cytoskeleton</td>
<td>Always has a cytoskeleton</td>
</tr>
<tr>
<td>Use binary fission for cell division</td>
<td>Use mitosis or meiosis for cell division</td>
</tr>
<tr>
<td>Reproduction is always asexual</td>
<td>Reproduction is either sexual or asexual</td>
</tr>
</tbody>
</table>

2.2.1 Human cervical cancer cells (HeLa cells)

HeLa cells are derived from cervical cells which belong to eukaryotic cells type. They are the main focus in exploring cancer related studies and were first known in 1951. They were taken from a cancer patient, named Henrietta Lacks, who passed away from cancer. She passed away due to cervical cancer in eight months; however samples of her cells were preserved in various laboratories for further research. The first human cells known to be continuously grown in culture are HeLa cells. HeLa cells have the capability of growing and division continuously and indefinitely, as long as suitable
environment is provided. This made it easy to literally immortalize the cells of Henrietta Lacks.

In early decades of cancer research, many researchers studied and explored the characteristics of HeLa cells, because the cells were easily available due to its continuous culture. A distinctive feature of HeLa cells and many other cultured cancer cells is that they are very adaptable and they can survive in circumstances where other cells would die. HeLa cells are so potent that they have the ability to occasionally contaminate other cell lines as well. HeLa cells are shown in figure 2.3.

![Figure 2.3: Cervical cancer cells (HeLa cells) (scale bar 50μm)](image)

### 2.2.2 Membrane of living cells

The cell membrane or plasma membrane is composed of a lipid bilayer. The cell membrane contains the cell cytoplasm and are found on all living cells. The cell membrane is shown in figure 2.4. The function of the cell membrane is to be selectively permeable to particular chemicals that can pass in and out of cells. It is also an interlocking surface that binds cells together. The cell membrane is only about 10nm thick. There are two part molecules called phospholipids which compose the layers. Hydrophilic phosphate heads facing outwards, and their non-polar, hydrophobic fatty acid tails facing each other in the middle of the bilayer. The lipids (fatty acids) are a hydrophobic layer that acts as a barrier to all but the smallest molecules, effectively isolating the two sides of the membrane.
2.3 Electroporation (EP)

Electroporation also called Electroperemeabilization is the use of high pulse electric field to modify the permeability of a cell membrane. This modification in permeability is achieved by using an electric field pulse to induce microscopic ‘pores’ in the cell membrane. These pores are commonly called ‘electropores’ that is why the process is commonly referred to as electroporation.

Many biotechnological applications and research requires transport of macromolecules such as genes, antibodies, and chemical drugs, into a host cell. For any particular application, a given transfer process selection is based on its efficacy, ease of use and side effects. A characteristic shared by most of the chemical and biological techniques is that they are usually cell-type dependent and have relatively poor efficiencies. Therefore, both versatile and efficient methods are being investigated. Electroporation was first reported in 1982 (Neumann et al., 1982) and is one of the methods reported to be effective for such delivery. Since its inception, this method has been a valuable tool for in-vitro delivery of small and large molecules into a large variety of cells. During this time, electroporation has been performed on living plants, animals, and humans (in-vivo electroporation), with an increasing focus on therapeutic uses (Dev et al., 2000; Smith et al., 2000; Muramatsu et al., 1998).
2.3.1. Overview of electroporation

Electroporation is a technique in which strong, rapid electric pulses are used to make the membrane of the living cell permeable to chemical species, which otherwise cannot cross the membrane (Weaver et al., 1996). Figure 2.5 demonstrates the process of electroporation. It’s a malleable and nontoxic physical technique which normally requires 1-4kV/cm electric pulse strength for 1µs till few ms in order to make the transient permeation possible through the cell membrane (Isambert, 1998). Despite the fact that the mechanism of electroporation is not fully explored, this technique has been used in biotechnology to incorporate different molecules into many different types of cells. Few examples of which are the incorporation of DNA and RNA fragments, proteins, antibodies and drugs into bacteria, yeasts, plant and mammalian cells (Chang et al., 1992; Neumann et al., 1989).

![Figure 2.5: Schematic diagram of electroporation process (Valero, 2006).](image)

The technique of the electric alteration of cell membrane conductivity is widely known since 1940 (Cole, 1972). Sale and Hamilton (Sale et al., 1967, 1968) stated that the subjection of cells to high electric field pulses leads to cell lysis. They described that the cell membranes were impaired by the transmembrane potential induced by the applied electric field.

The transmembrane potential $V_m$ was estimated from the equation, often referred to as the (steady-state) Schwan's equation (Schwan, 1957).

$$V_m = \frac{3}{2}Ea\cos \theta$$  \hspace{1cm} (2.1)

Where $E$ is the applied external electric field, $a$ is the radius of the cell, and $\theta$ is the angle between the direction of the field and the normal to the cell surface. The
critical transmembrane potential built up for electroporation to occur was found to be about ±1V. The phenomenon was called `electric breakdown' by Sale and Hamilton (Sale & Hamilton 1968).

Introduction of high electric fields to cells for long time compared to the membranes charging time is approximately one microsecond for the plasma membrane of mammalian cells (Weaver, 2000). This yields in charge redistribution, and making of a transmembrane voltage addition to the resting voltage. When the transmembrane voltage increases a few hundred millivolts, structural reconstruction starts. This makes the membrane to be instable. The shape of membrane then changes and makes aqueous paths. These are nano-scalable pathways which can provide entrance to foreign materials (Chang et al., 1992) and the membrane quickly attains some type of new electrical conduction pathways. Mass transfer can now happen using these pores with electrical control systems.

There are a number of early reports on the effects of electric fields on the living cell membrane. In 1958, the nodes of Ranvier of nerves were reported to be involved in some type of ‘breakdown’ (Stampfli, 1958). A decade later, in the late 1960s, Sale and Hamilton reported the damaging effects of strong electric fields on microorganisms and erythrocytes, suggesting non thermal membrane interactions (Hamilton et al., 1967; Sale et al., 1968). It was first found in the early 1970s that an induced electric field causes dielectric breakdown of the cell membrane and release intracellular components (Crowley, 1973; Zimmermann et al., 1974).

In 1970s reconstruction of membrane was discovered which would seal itself after creating pores (Kinosita et al., 1977; Gauger et al., 1979). By varying pulse parameters it was discovered that the introduced electric field could either have no effect on the cell membrane permeability, which causes reversible permeabilization of the cell membrane. On the other hand it can cause irreversible cell membrane porosity which damages the cells (Benz et al., 1979; Ho et al., 1996). Further evidence for chemical transport through membranes was observed in experiments with red blood cells (Zimmermann et al., 1976; Sukhorukov et al., 1998). Erythrocytes were also used to show DNA transfer into a cell induced with dielectric opening of the cell membrane pores (Auer et al., 1976). The cell transfection in-vitro through DNA electro-transfer were first experimentally shown by Neumann et al. and Wong and Neumann in 1982 (Neumann et al., 1982; Wong et al., 1982). From the start of nineties application of
electric field is frequently used technique in biotechnology and genetic engineering (Chang et al., 1992).

Crowley demonstrated membrane breakdown using electromechanical instability theory (Crowley, 1973). Crowley proposed that the electrostatic compressing force causes reduction in membrane thickness. The membrane area increase because the bilayer volume cannot be compressed, which forms lipids of increased wedge shaped. Thus, it can be concluded that phase equilibrium shift toward non-lamellar phase’s leads to destabilization of bilayer which results in membrane breakdown (Crowley, 1973; Rubinsky, 2004). However experimental evidence showed that Crowley’s theory failed to differentiate between reversible membrane breakdowns and irreversible membrane rupture. Bryant, Wolfe and Wilhelm et al. introduced theories that stated that mechanical stress causes membrane breakdown. Bryant and Wolfe stated that cell lysis do not occur due to electric field produced in the membrane but it occurs due to the isotropic mechanical surface tension formed in distorting the cell (Bryant et al., 1987). Wilhelm et al. demonstrated membrane breakdown by relating models of pore formation with those of mechanical stress (Wilhelm et al., 1993).

The knowledge of fusion cells using high-voltage electric pulses gained the attraction of more scholars among cell biologists and biophysicists towards electroporation (EP). Demange et al., (2011) introduced Giant cells viability by simply electro-pulsing a suspension of cells (Demange et al., 2011). Later, it was found that dielectrophoresis can be used to get interaction among cells (Manaresi et al., 2003). Dielectrophoresis can be known as the movement of somewhat non conducting particles or charged cells in non-uniform alternating electric fields (Manaresi et al., 2003). The cells can aggregate into chain lie structures when many particles are present in an alternating electric field. To achieve this particle size, particle density, electric field magnitude and frequency need to be optimized.

Alien genes are transfected into eukaryotic cells by introducing electric fields (Tsong & SU, 1999). This transfection process opens the pores of the plasma membrane of cells to intake the proteins which include antibodies or genetic material. From experimentation it was also seen that transfected genes can be obtained from the host cells (Tsong & SU, 1999). Similarly, electroporation was recognized as an efficient method for the introduction of foreign molecules into cells of any basis (Lavitranos, 1989).
Different experiments were performed in 1998 on human skin fibroblast and it was observed that most resourceful transient chloramphenicol acetyltransferase expression was detected after transfection with plasmid (Brown, 1998). The point that these cells show transfected exogenous DNA properties could conclude in many important applications in the study of human genetic diseases and cancer. Others researches observed that the electroporation of the skin could be used to enhance transdermal drug delivery (Henry et al., 1998).

In 1996, it was observed that embedding of the protein in cell plasma membrane was potentially available by introducing electric fields pulses on a suspension of cells in the presence of a specific membrane protein (Zimmermann, 1996). This process is called as electroinsertion. In further research, electroporation of excitable membranes was studied (Chang & Reese, 1990). The morphological indication showed that electrically induced membrane breakdown of remote cardiomyocytes cells has signs of the presence of protein (Weaver & Chizmadzhev, 1996).

The electroporation technique is explained in many studies by combining the theories of pre-existing pores, defects or fluctuations in the cell membrane integrity. Neumann and Boldt stated that electroporation technique is a transition phase from hydrophobic pathways to hydrophilic pathways in the lipid layers (Neumann et al., 1990). Similarly, Chernomordik reported that it is small hydrophobic pores or defects, which later becomes large in size and hydrophilic when subjected to electric field (Chernomordik, 1992). The models of denaturation shows that the membrane can be permeated at the protein channels, where it experiences denaturation after subjected to electrical modification or Joule heating (Tsong, 1992).

Weaver and Powell reported that electropores are dynamic and transient structures in the lipid bilayer membrane (Weaver et al., 1989). In Weaver and Powell’s model either small pores already exist in the membrane or created by electric field in the membrane. The electrical pulse generates electric potential which expands these tiny pores. In order to investigate the pore population rate in a membrane and the number of electropores, Weaver and Barnett established pore population models (Weaver et al., 1992). If the energy is expended for edge formation and is taken by increasing pore areas the electropores will remain stable. According to Weaver’s model, these stable pores acts as passageways for external substances to pass into the
cells. Conversely, the existence of these pores has never been reported in the membrane of mammalian cells.

Various methods for electroporation have been established in different areas including genetics, immunology, microbiology, biochemistry, medicine, pharmacology, and toxicology. While in case of in-vivo introduction of pulsed electric field to allow molecular entrance is gaining attention for multiple applications (Somiari, 2000).

Electrochemotherapy is an application of electroporation targets cancer cells. Short duration high electric field is introduced just enough to target tumour cells. Once the membrane is open anticancer materials can be introduced in the cells to treat cancer. For now, these methods are used in in-vivo environments (Somiari, 2000). Keeping importance of this application, this study focuses on the difficulties faced in the experiments. This research also presents better understanding of the process by presenting optimization of parameters for electroporation process.

2.3.1.1 Basic circuit for electroporation

Figure 2.6 presents the basic circuit setup of the electroporation apparatus. The typical process of electroporation circuit is controlled by switch 1 and switch 2. The capacitor is charged by the closing the switch 1 and the capacitor stores a high voltage of around 10-100kV/cm. Once the second switch is closed, this voltage discharges through the liquid of the cell suspension. The pulse DC is thought both to disrupt temporarily the cell membrane and allow DNA into cells.
A method for enhancing electroporation involves the application of a short electric pulse that transiently disrupts cellular membranes (Chang et al., 1992). The electric field is typically applied as one or more short (μs to ms) pulse with a rectangular or exponential decay waveform. The increase of cell permeability is thought to be due to the formation of short lived aqueous pathways “pores” in the plasma membrane. It depends upon the field strength, length, and number of pulses (Weaver, 2000). The most important parameters for effective electroporation are electric field strength and the pulse length (Dower et al., 1988).

Figure 2.6: Basic circuit diagram of the electroporation setup

2.3.1.2 Method for enhanced electroporation
In order to electroporate the cell membrane a transmembrane potential difference of between 0.7V - 1V needs to achieved. The required field strength $E$ is inversely proportional to the cell diameter.

High field strength and generator pulse length enhance the permeability of the cell membrane. Applying too high a field strength can result in the cell being unable to repair itself. On the other hand, if the field strength is too low, the breakdown of the cell membrane may not be achieved. If the pulse length is too short, the pore of the cell membrane will not be opened.

2.3.1.3 Electroporation of suspension

Most researchers have demonstrated that electroporation can be successfully applied to different types of mammalian cells (Chakrabarti et al., 1989; Gilbert et al., 1987; Jordan et al., 2004), yeast (Wall et al., 2004), bacteria (Lee et al., 2002) and red blood cells (Mouneimne et al., 1990). However, the electroporation is not meaningful for cells e.g. epithelial cells, because it poorly mimics in-vivo cell function and geometry found in tissues (Gharter, 2004).
2.3.2. Electroporation types

A process in which high electric field is applied to cells to open the membrane of cells of a small time is known as reversible. Reversible electroporation allows introduction of foreign molecules or genes into cells and change cells properties which can not be achieved in natural conditions. In biomedical and medical sciences, alien molecules and other agents to cells are extensively used specially in case of reversible electroporation. Cells fusion is also one of the applications of reversible electroporation (Nazib et al., 2013). This method has been used to induce external substance, for example the introduction of exogenous DNA into cells. For the eradication of cancer cells this method can also be used and referred as electrochemotherapy (Jaroszeski et al., 1997). Irreversible electroporation on the other hand is mostly used to kill cells. In this process and electric field is introduced which is higher than what cell’s structure can bear. This leads to the death of cells. Microbial deactivation is a process in which this technique was used to ill bacterial cells (Schenk & Laddaga, 1992).

2.3.3. Electroporation parameters

Two of the main parameters for electroporation process are applied electric field strength and pulse duration of applied electric field. Other type of parameters also plays their role in efficient electroporation. Other environmental and chemical conditions can also increase or decrease the intake foreign material. Figure 2.8 shows the correlation of pulse duration and electric field strength in electroporation.
Figure 2.8 shows that, the poration do not occur if the strength of electric field and range of pulse duration is low. When the intensity of electric field or the exposure of pulse duration increases, it reaches to a certain range where much clear effects are expected, even though if the variation of temperature is acceptable. If the pulse electric filed strength and pulse duration increases to a certain value, the phenomenon of cell lysis can occur, this means that the cells under exposure could be killed.

The preferred range of operation for medical applications is long time duration pulses and low-electric voltage as shown in the right side of Figure 2.8. The Figure shows that gene transfection occurs in the range of pulse durations and electric field amplitudes. Drug delivery needs shorter pulses and higher electric fields. One microsecond range of pulse duration is required for bacterial decontamination subjected to electric fields from 10kV/cm to 100kV/cm. A completely different level of applications can be reported when the values of graph travels to short pulse duration with very high-electric voltage. With increasing pulse duration the membrane charging time, the subcellular effects contribute to the intracellular electromanipulation instead of plasma membrane electroporation.
2.4 Experimental studies

Considering theoretical techniques, different experimental setups can be used to carry out studies for these theories. Electroporation parameters play vital role which can be observed with experimental setup for best results.

In experimental work, it is shown that higher magnitude of external force which is electric field increases the permeability of cells five to six times when compared to moderate electric field application (Esser et al., 2007; Jiang et al., 2015).

The electroporation effect causes pores formation on the membrane by making liquefied passages from which external substance can travel inside the cells. However, if electric field strength increases the bearing properties of membrane it causes irreversible electroporation causing ruptures which eventually kills the cells (Esser et al., 2007; Jiang et al., 2015). Moreover, the pores reseal and conductivity of the membrane increases (Jiang et al., 2015).

Considering potential applications of these techniques, extensive experimental work has been conducted to understand electroporation in detail. Some researchers conducted the study of electric current through membrane while electroporation and its relation to porosity while others focused on time duration of voltage applied (Chung et al., 1998; Sinton & Cuevas, 1996). Fluorescent molecules have also been focused by many researchers.

Since the size of the pores is extremely small usually in nanometres and also their formation and growth rate is high. Therefore, it is difficult to observe the process of electroporation directly. Even though many researchers succeeded in providing substantial amount of progress, still there are various basic aspects of electroporation that needs further exploration and understanding (Rulong et al., 2014).

So far researchers have provided many techniques in order to find out the effects of electroporation on biological cells. This research focuses to explain experimentation technique. The development of this experimental setup will discuss and explain the best applications of electroporation.

The study of cells and biological materials outside their naturally occurring environment is called In-vitro. This is the most commonly used techniques in electroporation for analysis of cell and biological material behaviour (Hatanaka & Murakami 2002; El-Ali et al., 2006). To make cells survive outside their naturally
occurring environment, an artificial environment is created to make the cells survive and act naturally.

The artificial environment includes the control of temperature, CO₂ level and humidity. It makes the setup flexible to study the effect of not only externally applied electric field but also artificially maintained environment for electroporation (Davis & Warren, 1994). The parameters are important factor for quantitative researches. On the other hand, it is vital to understand that these experiments are in-vivo which has artificial environments outside the actual biological body. These techniques cannot be applied on complicated body systems. Hence, it is crucial to understand the outcome from these experiments and cannot be directly applied on complex biological systems.

2.5 Electroporation applications

Electroporation can be beneficial in many ways in molecular biology, biochemistry, medicine and other biological research.

2.5.1 Electrochemotherapy (ECT)

In cancer chemotherapy, some drugs do not exhibit anti-tumour effects because of insufficient transport through the cell membrane (Miklavcic et al., 2004). A combined use of chemotherapeutic drugs and application of electric pulses is known as Electrochemotherapy and is useful for local tumour control. Especially, Bleomycin has been reported to have shown a 700-fold increased cytotoxicity when used in ECT (Cemazar et al., 2010; Sersa et al., 2008). This helps to achieve a substantial anti-tumour effect with a small amount of drug that limits its side effects. Bleomycin and cisplatin have proven to be much more effective in electrochemotherapy than in standard chemotherapy when applied to tumour cell lines in-vitro, as well as in-vivo on tumours in mice (Mir et al., 1991, 1995). Clinical trials have been carried out with encouraging results (Gothelf et al., 2003; Tozon et al., 2005; Snoj et al., 2005).
2.5.2 Electrogenetransfection (EGT)

Electroporation transfer DNA into cells to alter some form of gene therapy, usually known as Electrogenetransfection. It is currently being used in various pre-clinical trials (Mir, 2001). As a non-viral technique, Electrogenetransfection has large potential to transfer genetic material into cells. This process focuses on treating genetic diseases (Budak et al., 2005; Bertino, 2008).

Figure 2.9 shows the process of DNA transfection using electroporation. In the first part DNA is introduced which binds with the border of cell as shown in Figure 2.9 (a). Once DNA is introduced, electroporation is performed which opens cell pores and DNA entrance into the cell as shows in Figure 2.9 (b). Figure 2.9 (c) shows DNA inside the cell after electroporation.

![Figure 2.9: Cell electrotransfection process](image)

2.5.3 Electrofusion (EF)

Application of electric pulses can lead to fusion of membrane in close-contact adjacent cells. Electrofusion can be used to create genetic hybrids and in order to encapsulate both original cells intracellular material within a single enclosed membrane (Zimmermann, 1982). The fusion of tumour cells with an antibody secreting stimulated B-lymphocytes forms a hybrid cell known as Hybridoma. Later on, this hybridoma has the ability to continue their growth in a culture environment, and an enormous amount of desired antibodies can be obtained. Electrofusion is a beneficial
Fusion of cells by electrofusion is a multiple-stage process that involves fusion of lipid and mixing of cytoplasmic content. Figure 2.10 shows the process of electrofusion of two cells. When two cells are introduced to electroporation, the cells start to porate. In the first stage, since the cell membrane is porous, they tend to join, making the fusion of outer layer but the cytoplasmic content is separate. At this stage, the internal structure of cells is independent and only the outer membrane is merged to make a common enveloping lipid. In the second stage, the fusion occurs in both monolayers of lipid bilayer. This takes the fusion process of the two cells further in their structures. Once both fusion of monolayers occurs, the cytoskeletal networks of two cells will gradually merge. Once all the content is merged, the two cells will appear to be one cell with complete and shared cytoplasm.

![Cell electrofusion process](image)

**Figure 2.10: Cell electrofusion process**

### 2.5.4 Transdermal drug delivery (TDD)

The transdermal drug delivery is potentially valuable because the technology can be site-specific, good control of the dose and located outside the body. In order to deliver drugs through the skin by means of electrical pulses, the stratum corneum with the thickness of 20μm must be electroporate. Because its thickness corresponds to approximately 200 times of the lipid bilayer membranes thickness, the required potential difference across the stratum corneum is in the order of 200V.
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