OPTIMIZATION OF LOW AMPLITUDE PULSE ELECTRIC FIELD EXPOSURE ON COLON CANCER CELL FOR WOUND HEALING APPLICATION

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UNIVERSITI TUN HUSSEIN ONN MALAYSIA
OPTIMIZATION OF LOW AMPLITUDE PULSE ELECTRIC FIELD EXPOSURE ON COLON CANCER CELL FOR WOUND HEALING APPLICATION

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A thesis submitted in
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For my father, Alhaji Hassan Mamman, my mother, Fatima Musa, my wife, Ramat Aliu, and my children, Shukura Buhari Hassan, Abdurrahman Buhari Hassan, Amaturrahman Buhari Hassan and Siddiqa Buhari Hassan.
Thanks are to ALLAH the most beneficent and most merciful. I thank ALLAH for given me the life, health, wealth, power and wisdom to achieve this milestone in my life. May Allah in his mercy make this research beneficial to the entire mankind.

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ABSTRACT

Pulse electric field (PEF) is a method of creating transient pores in the cell membrane by exposing it to a high voltage electric field of short duration. Beside having the effect of opening pores in the membrane, PEF can change the cytoskeletal restructuring of the cell, which has an impact on the cell morphological properties and signalling pathways. There was evidence that extracellular matrix (ECM) protein and transforming growth factor beta (TGF-β) can influence cellular behaviours. Similarly, PEF was also found to modulate the cellular behaviours. However, a combined influence of PEF and ECM on cellular behaviour which could further enhances the cellular processes for wound healing and tissue engineering application has not yet been investigated. Therefore, the aim of this study is to examine the effect of PEF in combination with ECM or TGF-βs on cellular behaviour such as attachment proliferation, adhesion, migration and alignment of HT29 cell line monolayer in vitro. Cell-surface adhesion was studied via trypsinization assay in which the time taken for cells to detach from the monolayer would give the means of assessing the strength of adhesion. Cell migration was studied by creating a wound model on a confluence monolayer of the cell in vitro. Cell alignment was investigated via micro contact printing techniques. The PEF was found to increase the attachment, proliferation and migration rate of the HT29 cell by 67.6, 27.1 and 108% respectively and decrease the adhesion strength of the cells by 29.2%. The results also show that 600V/cm and 5ms electric parameter could potentially reduce the unpleasant sensation associated with the standard electric field parameter currently in used in electrochemotherapy. The results of the cell alignment to the ECM proteins pattern have shown that PEF has improved the HT29 cell alignment by more than 40%. Similarly, the PEF was found to increase the proliferation and decrease the adhesion rate of cells in a TGF-β3 supplement by 50.2 and 24.1% respectively. Thus, the outcomes of this research revealed that the use of PEF in the presence of ECM protein have potential implication in wound healing application (by increasing the rate of cell migration and decreasing cell adhesion).
**ABSTRAK**

Medan Elektrik Denyut (PEF) adalah satu kaedah untuk menghasilkan liang sementara dalam sel membran dengan cara mendedahkan sel-sel tersebut kepada medan elektrik bervoltan tinggi dalam jangka masa yang pendek. Selain daripada memberi kesan pembukaan liang membran, PEF boleh mengubah penstrukturan semula sitoskeleton sel, di mana ianya akan memberi kesan terhadap sifat morfologi dan laluan isyarat bagi sel tersebut. Terdapat bukti bahawa matriks extra-selular (ECM) protein dan faktor perubahan pertumbuhan beta (TGF-β) boleh mempengaruhi sifat sel. Tambahan pula, PEF juga didapati dapat memodulasi sifat sel itu sendiri. Walau bagaimanapun, kesan gabungan PEF dan ECM pada sifat sel yang dapat meningkatkan lagi proses-proses sel bagi tujuan aplikasi dalam kejuruteraan tisu masih belum dikaji secara mendalam. Justeru, tujuan kajian ini adalah untuk menyelidik kesan penggabungan teknik PEF dan ECM atau TGF-βs pada sifat sel seperti proses percambahan, pelekat sel, penghijrahan dan penjajaran baris sel HT29 lapisan-mono dalam kaedah *in vitro*. Proses pelekat sel pada permukaan ini dikaji melalui proses tripsinasi di mana tempoh masa yang diambil kira untuk melihat sel-sel melakukan proses penanggalan daripada lapisan-mono akan memberikan jawapan terhadap proses dalam mengkaji kekuatan pelekat sel. Proses penghijrahan sel telah dikaji dengan menghasilkan satu model luka pada sel mono-lapisan dalam kaedah *in vitro*. Proses penjajaran sel telah dikaji melalui teknik percetakan mikro kontak. Hasilnya, PEF didapati dapat meningkatkan kadar percambahan dan penjajaran sel HT29 dengan kadar 67.6, 27.1 dan 108% dan telah mengurangkan kekuatan pelekat sel sebanyak 29.2%. Hasil kajian ini juga mendapati bahawa 600V/cm dan 5ms parameter elektrik boleh digunakan untuk mengurangkan atau menghapuskan perkara yang tidak diingini berkaitan dengan parameter standard elektrik yang sedang digunakan dalam rawatan elektrokemoterapi. Keputusan teknik penjajaran sel dalam bentuk protein ECM telah menunjukkan bahawa PEF telah meningkatkan hasil penjajaran sel HT29 sebanyak lebih daripada 40%. Begitu juga, PEF didapati dapat meningkatkan percambahan dan mengurangkan kadar pelekat sel dalam suplemen TGF-β3 50.2 dan 24.1%. Oleh itu, hasil kajian ini berpotensi memberikan impak dalam penyembuhan luka dan dalam aplikasi kejuruteraan tisu apabila dikaji dengan lebih mendalam pada masa akan datang.
CONTENTS

TITLE i
DECLARATION ii
DEDICATION iii
ACKNOWLEDGEMENT iv
ABSTRACT v
ABSTRAK vi
CONTENTS vii
LIST OF TABLES xiii
LIST OF FIGURES xvi
LIST OF SYMBOLS AND ABBREVIATIONS xxii
LIST OF APPENDICES xxiii
LIST OF PUBLICATIONS xxiv

CHAPTER 1 INTRODUCTION 1
  1.1 Background information 1
  1.2 Problem statement 5
  1.3 Aim and objectives of the research 6
  1.4 Scopes of the study 7
  1.5 Thesis structure 7
  1.6 Contributions of the study 8

CHAPTER 2 LITERATURE REVIEW 10
  2.1 The cell 10
    2.1.1 HT29 cell line 12
    2.1.2 The cell membrane 12
    2.1.3 Membrane voltage 13
    2.1.4 Cell cycle 14
    2.1.5 Cell adhesion 15
2.2 Extracellular matrix (ECM) protein 16
2.3 Cell signalling 16
2.4 Cell model 17
2.5 Theory of electroporation 18
  2.5.1 Stages of electroporation 19
  2.5.2 Parameters affecting electroporation 20
  2.5.3 Application of electroporation 20
    2.5.3.1 Electrofusion 20
    2.5.3.2 Electrochemotherapy 21
    2.5.3.3 Electrogenetherapy 21
    2.5.3.4 Electroinsertion 22
    2.5.3.5 Electro-sterilisation 22
  2.5.4 Pulse electric field effect and cell injury 22
  2.6 Electric current correlation with body sensation 23
  2.7 Cell viability 23
  2.8 Microcontact printing technique 24
  2.9 Extracellular matrix protein as a tool for microcontact printing technique 24
    2.10.1 Laminin 25
    2.10.2 Fibronectin 25
    2.10.3 Collagen 26
  2.10 Previous studies on cell guidance on ECM pattern via MCP technique 26
  2.11 Transforming growth factors beta 28
  2.12 Previous studies on the effect of transforming growth factors beta on cellular behaviours 29
  2.13 Cell adhesion, migration and electric field as related to wound healing process 31
  2.14 Summary 32

CHAPTER 3 MATERIALS AND METHODS 34
3.1 Introduction 34
3.2 Material 37
  3.2.1 Reagents 37
3.3 Equipment 39
CHAPTER 3 METHODS

3.4 Methods

3.4.1 Precautionary measures

3.4.2 Cell culture protocol

3.4.2.1 Thawing the HT29 cell line

3.4.2.2 Propagating the HT29 cell line

3.4.2.3 Cell subculture

3.4.3 Cell detachment or trypsinization

3.4.4 Cell counting with Haemocytometer

3.4.5 Determination of viability percentage

3.5 Integrated devices for time-lapse live imaging

3.5.1 Chamlide TC incubation systems

3.5.2 TS100 Nikon inverted microscope

3.5.3 DinoCapture2.0 software

3.6 Electroporation

3.7 Statistical analysis

3.8 Summary

CHAPTER 4 OPTIMIZATION OF ELECTRIC FIELD PARAMETERS FOR HT29 CELL LINE

4.1 Introduction

4.2 Material and methods

4.2.1 Cell culture

4.2.2 Electroporation

4.2.3 Measurement of cell proliferation

4.2.4 Measurement of cell length

4.3 Results and discussion

4.3.1 Measurement of cell proliferation

4.3.2 Measurement of cell length

4.4 Summary

CHAPTER 5 INFLUENCE OF ELECTROPORATION ON CELL ATTACHMENT, PROLIFERATION, ADHESION AND MIGRATION PROPERTIES OF HT29 CELL LINE

5.1 Introduction

5.2 Materials and methods

5.2.1 Cell culture
CHAPTER 7 STUDYING THE INFLUENCE OF ELECTROPORATION ON HT29 CELL LINE INTERACTION WITH MICRO-PATTERNED EXTRACELLULAR MATRIX PROTEIN

7.1 Introduction
7.2 Materials and methods
  7.2.1 Cell culture
  7.2.2 MCP technique
  7.2.3 Protocol for cell electroporation
  7.2.4 Protocol of plating cells on glass coverslips
7.3 Results and discussion
  7.3.1 Stamp construction and micro-contact pattern printing
  7.3.2 Measurement of cell alignment
  7.3.3 Measurement of cell elongation or cell length
  7.3.4 Influence of PEF on cell alignment and cell elongation
7.4 Summary

CHAPTER 8 INVESTIGATION OF COMBINED EFFECT OF PEF AND TGF-β3 ON PROLIFERATION, SPREADING AND ADHESION OF HT29 CELL LINE

8.1 Introduction
  8.1.1 Transforming growth factor beta-3
8.2 Materials and methods
  8.2.1 Reconstitution of TGF-β3 solution
  8.2.2 Cell culture
  8.2.3 Electroporation
  8.2.4 Cell plating
  8.2.5 Cell proliferation analysis
  8.2.6 Cell length measurement
  8.2.7 Cell detachment analysis
8.3 Results and discussion
  8.3.1 Cell proliferation
  8.3.2 Cell length measurement
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>HT29 properties</td>
<td>12</td>
</tr>
<tr>
<td>2.2</td>
<td>Previous studies on cell alignment onto ECM protein pattern via MCP techniques</td>
<td>27</td>
</tr>
<tr>
<td>3.1</td>
<td>The ECM 830 BTX electroporator electrical characteristic and the technical specifications</td>
<td>41</td>
</tr>
<tr>
<td>4.1</td>
<td>High voltage mode variation of maximum pulse duration with voltage</td>
<td>57</td>
</tr>
<tr>
<td>4.2</td>
<td>Electric field parameter used and actual value delivered</td>
<td>59</td>
</tr>
<tr>
<td>4.3</td>
<td>Proliferation rate of HT29 cell line treated with various electric field parameters</td>
<td>61</td>
</tr>
<tr>
<td>4.4</td>
<td>Statistical analysis of HT29 cell line proliferation factor for different electric field parameters</td>
<td>63</td>
</tr>
<tr>
<td>4.5</td>
<td>HT29 cell line proliferation rate at fixed pulse amplitude of 600V/cm and different pulse duration, after 48 hours in culture</td>
<td>65</td>
</tr>
<tr>
<td>4.6</td>
<td>Statistical analysis of HT29 cell line proliferation factor for different pulse duration at fixed amplitude of 600V/cm</td>
<td>65</td>
</tr>
<tr>
<td>4.7</td>
<td>Cell length measurement of HT29 cell line treated with various electric field parameters</td>
<td>66</td>
</tr>
<tr>
<td>4.8</td>
<td>Statistical analysis of HT29 cell length for different electric field parameters</td>
<td>68</td>
</tr>
<tr>
<td>4.9</td>
<td>HT29 cell length at fixed pulse amplitude of 600V/cm and different pulse duration, after 48 hours in culture</td>
<td>70</td>
</tr>
<tr>
<td>5.1</td>
<td>Average length of EP and NEP HT29 cell line</td>
<td>82</td>
</tr>
<tr>
<td>5.2</td>
<td>P-values from statistical analysis of proliferation percentage over time</td>
<td>84</td>
</tr>
</tbody>
</table>
5.3 Average confluence percentage of HT29 cell lines over time ± Standard error of the mean
5.4 Average length of HT29 cell line over time
5.5 Average wound distance over time for EP and NEP cells
5.6 Migration speed of HT29 cells line over time, for both EP cells and NEP cells
6.1 Mean permeability percentage of HT29 cell line after electroporation with different electric field parameters
6.2 Statistical analysis of permeabilization percentage of HT29 cell line
6.3 HT29 cell line viability percentage after 24 hours of treatment with various electric field parameters
6.4 Details of pairwise comparison of P–values obtained from HT29 cell line viability percentage
7.1 MCP process on glass coverslip with the ECM protein
7.2 Angle of alignment for HT29 cell line seeded on coverslip micro-contact printed with ECM protein after 48 hours of seeding
7.3 Statistical analysis of the angle of alignment between the different proteins
7.4 Statistical analysis of angle of alignment of the different stamp sizes on the same protein pattern
7.5 Average length of HT29 cell line seeded on 25, 50, 100µm micro-contact printed substrate and the control substrate after 48 hours
7.6 Statistical analysis of cell elongation between the different proteins used on the same stamp size
7.7 Statistical analysis of cell elongation on the different stamp sizes on the same protein pattern
7.8 PEF treated HT29 cell line alignment on 100, 50 and 50µm micro-contact printed substrates of laminin, fibronectin and collagen
7.9 Length of PEF treated HT29 cell line on 100, 50 and 50µm micro-contact printed substrates of laminin, fibronectin and collagen

8.1 Specification of TGF-β3

8.2 Proliferation percentage of HT29 cell line seeded in different supplemented media after 72 hours of seeding

8.3 Statistical analysis of the cell proliferation percentage of HT29 cell line seeded in different supplemented media for 72 hours

8.4 Proliferation percentage of HT29 cell line treated with PEF and seeded in different supplemented media after 72 hours in culture

8.5 Statistical analysis of the cell proliferation percentage of HT29 cell line treated with PEF and seeded in different supplemented media for 72 hours

8.6 Statistical analysis of proliferation percentage between PEF treated and non-treated HT29 cell line in the different supplemented media

8.7 Average cell length of HT29 cell line versus time, seeded in different supplemented media

8.8 Average cell length of HT29 cell line versus time, treated with EP and seeded in different supplemented media

8.9 Cell detachment percentage of HT29 cell line with and without EP exposure seeded in different supplemented media
LIST OF FIGURES

1.1 Cell exposed to an external electric field. (A) No effect, because the field is too small to cause permeabilization. (B) Cell reseal after electroporation. (C) The membrane is damaged due to high field intensity irreversible electroporation (Zupanic et al., 2012) 3

2.1 A typical diagram of a prokaryotic cell 11
2.2 A typical diagram of a eukaryotic cell (Animal cell) 11
2.3 A diagram of a cell membrane 13
2.4 Cell cycle diagram 15
3.1 Flow chart representation of the general methodology 35
3.2 Image representation of various reagent utilized in the research (a) RPMI1640 medium (b) Fetal bovine serum (c) Pen/strep-Antibiotic (d) Phosphate buffer saline (e) Tryple express (f) Trypan blue (g) Propidium Iodide 37
3.3 Image representation of various equipment utilized in the research (a) Biosafety hood (b) Incubator (c) Water bath (d) Centrifuge machine (e) Refrigerator (f) Freezer 39
3.4 The ECM 830 BTX electroporator 41
3.5 A Haemocytometer schematic layout (Rodamporn, 2010) 46
3.6 Integrated devices for live cell imaging 47
3.7 Chamlide incubation system components: (A) Chamlide incubator (B) CO₂ controller -FC-5 (C) Temperature controller -CU109 48
3.8 The EC-B25 magnetic stimulation chamber 48
3.9 Image of TS100 Nikon inverted microscope equipped with Dino camera 49
3.10 Measurement properties window in DinoCapture2.0 software 50
3.11 An example of line measurement with DinoCapture2.0 software 51
3.12 An example of angle measurement with DinoCapture2.0 software 52
3.13 An example of gridline measurement with DinoCapture2.0 software
4.1 HT29 proliferation factor for different electric field parameters
4.2 HT29 cell proliferation factor at fixed amplitude of 600V/cm and various pulse durations
4.3 HT29 cell length measurement for different EP parameters
5.1 Chamlide EC-B25 magnetic chamber
5.2 Chamlide live cell incubator (Mini incubator)
5.3 An inverted microscope equipped with digital camera and DinoCapture2.0 software
5.4 Component of Chamlide EC-B25 magnetic chamber (Live cell instrument Inc., 2011)
5.5 Phase contrast images showing the electroporated (EP) and non-electroporated (NEP) HT29 cells after 10, 20, 30, 40, 50 and 60 minutes respectively. (Scale bar = 30µm)
5.6 Measured average length of EP and NEP HT29 cells against time
5.7 Graph showing confluence percentage over time for both electroporated and non-electroporated HT29 cell lines
5.8 Graph showing average cell length over time for both electroporated and non-electroporated HT29 cell lines
5.9 Images of electroporated (EP) and non-electroporated HT29 cells at 0, 6, 24, 48, 64 and 72 hours respectively. (Scale bar = 100µm)
5.10 Trypsinization process of HT29 cell line under pulse electric treatment and control group. Scale bar = 50µm
5.11 Graph showing wound distance and closure rate for HT29 cell line
5.12 Phase contrast images of HT29 cells line during migration process with PEF treatment
5.13 Phase contrast images of HT29 cells line during migration process without PEF treatment
5.14 The plot of migration speed of HT29 cells lines over time
6.1 Images of fluorescent and phase contrast images of HT29 cell line after treatment with various electric field parameters. Scale bar = 100µm
6.2 Permeability percentage of HT29 cell line after electroporation with various electric field parameters 104
6.3 Graph showing viability percentage of HT29 cell line after 24 hours of treatment with various electric field parameters 106
6.4 Images of HT29 cell line during cell viability measurement 107
7.1 PDMS stamp constructed for the micro-contact printing study: (A) 25\(\mu m\); (B) 50\(\mu m\); and (C) 100\(\mu m\). Scale bar = 100\(\mu m\) 115
7.2 Images of glass coverslips micro-printed with 25, 50 and 100\(\mu m\) stamp width of laminin protein. Scale bar = 100\(\mu m\) 116
7.3 Images of glass coverslips micro-printed with 25, 50 and 100\(\mu m\) stamp width of fibronectin protein. Scale bar = 100\(\mu m\) 116
7.4 Images of glass coverslips micro-printed with 25, 50 and 100\(\mu m\) stamp width of collagen protein. Scale bar = 100\(\mu m\) 117
7.5 Images of HT29 cell line on glass coverslips micro-contact printed with the ECM proteins and the control after 48 hours. Scale bar =100\(\mu m\) 118
7.6 Graph comparing the angle of alignment of HT29 cell line seeded on 25, 50, 100\(\mu m\) micro-contact printed substrates and control substrate after 48 hours 121
7.7 Graph comparing the cell length of HT29 cell line seeded on 25, 50, 100\(\mu m\) micro-contact printed substrates and control substrate after 48 hours 124
7.8 Images of HT29 cell line on 100, 50 and 50\(\mu m\) micro-contact printed substrates of laminin, fibronectin and collagen respectively. Scale bar = 100\(\mu m\) 125
7.9 Graph showing PEF treated HT29 cell line alignment on 100, 50 and 50\(\mu m\) micro-contact printed substrates of laminin, fibronectin and collagen 127
7.10 Graph showing length of PEF treated HT29 cell line on 100, 50 and 50\(\mu m\) micro-contact printed substrates of laminin, fibronectin and collagen 127
8.1 Graph of proliferation percentage of HT29 cell line seeded in different supplemented media after 72 hours of seeding 138
8.2 Graph of average proliferation percentage of HT29 cell line treated with PEF and seeded in different supplemented media after 72 hours in culture

8.3 Graph of the average cell length of HT29 cell line versus time, seeded in different supplemented media

8.4 Graph of the average cell length of HT29 cell line versus time, treated with PEF and seeded in different supplemented media

8.5 Images of HT29 cell line plated on different supplemented media after 12 hours of seeding with and without PEF exposure. Scale bar = 50µm

8.6 Images of HT29 cell line plated on different supplemented media after 24 hours of seeding with and without PEF exposure. Scale bar = 50µm

8.7 Images of HT29 cell line plated on different supplemented media after 48 hours of seeding with and without PEF exposure. Scale bar = 50µm

8.8 Images of HT29 cell line plated on different supplemented media after 72 hours of seeding with and without PEF exposure. Scale bar = 50µm

8.9 Images of HT29 cell detachment in BSA supplemented media after 72 hours of seeding. Scale bar = 100µm

8.10 Images of HT29 cell detachment in BSA+PEF supplemented media after 72 hours of seeding. Scale bar = 100µm

8.11 Images of HT29 cell detachment in HCL supplemented media after 72 hours of seeding. Scale bar = 100µm

8.12 Images of HT29 cell detachment in HCL+PEF supplemented media after 72 hours of seeding. Scale bar = 100µm

8.13 Images of HT29 cell detachment in BSA/HCL supplemented media after 72 hours of seeding. Scale bar = 100µm

8.14 Images of HT29 cell detachment in BSA/HCL+PEF supplemented media after 72 hours of seeding. Scale bar = 100µm

8.15 Images of HT29 cell detachment in TGF-β3 supplemented media after 72 hours of seeding. Scale bar = 100µm
8.16 Images of HT29 cell detachment in TGF-β3+PEF supplemented media after 72 hours of seeding. Scale bar = 100µm

8.17 Images of HT29 cell detachment in control group (unsupplemented media) after 72 hours of seeding. Scale bar = 100µm
### LIST OF SYMBOLS AND ABBREVIATIONS

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>$cm$</td>
<td>Centimetre</td>
</tr>
<tr>
<td>$E$</td>
<td>Electric field strength</td>
</tr>
<tr>
<td>$F$</td>
<td>Farad</td>
</tr>
<tr>
<td>$ml$</td>
<td>Millilitre</td>
</tr>
<tr>
<td>$ms$</td>
<td>Millisecond</td>
</tr>
<tr>
<td>$nm$</td>
<td>Nanometre</td>
</tr>
<tr>
<td>$T$</td>
<td>Pulse duration</td>
</tr>
<tr>
<td>$V$</td>
<td>Voltage</td>
</tr>
<tr>
<td>$\mu l$</td>
<td>Microliter</td>
</tr>
<tr>
<td>$\mu s$</td>
<td>Microsecond</td>
</tr>
<tr>
<td>$^\circ C$</td>
<td>Degrees Celsius</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>BSA</td>
<td>Basal serum albumin</td>
</tr>
<tr>
<td>CCD</td>
<td>Charge-couple device</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EP</td>
<td>Electroporated</td>
</tr>
<tr>
<td>ECT</td>
<td>Electrochemotherapy</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>HCL</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>HV</td>
<td>High voltage</td>
</tr>
<tr>
<td>HT29</td>
<td>Colon cell line</td>
</tr>
<tr>
<td>IVD</td>
<td>In vitro diagnosis</td>
</tr>
<tr>
<td>LED</td>
<td>Light emitting diode</td>
</tr>
<tr>
<td>LV</td>
<td>Low voltage</td>
</tr>
<tr>
<td>MCP</td>
<td>Micro contact printing</td>
</tr>
<tr>
<td>MPW</td>
<td>Measurement properties window</td>
</tr>
</tbody>
</table>
**NEP** - Non-electroporated

**PBS** - Phosphate buffer saline

**PDMS** - Polydimethylsiloxane

**PEF** - Pulse electric field

**PI** - Propidium iodide

**RPMI** - Roswell Park Memorial Institute Media

**SD** - Standard deviation

**SPSS** - Statistical package for social sciences

**SEM** - Standard error in mean

**SEFPECT** - Standard electric field parameters for Electrochemotherapy

**TES** - Tryple express solution

**TGF-β3** - Transforming growth factor beta-3

**UTHM** - University Tun Hussein Onn Malaysia

**UV** - Ultra-violet
LIST OF APPENDICES

A  Published journals, conference papers and book chapters  184
LIST OF PUBLICATIONS

Conference Proceedings


**Journals**


**Book Chapters**


Book chapters submitted for publication.


Research Grant Award:

CHAPTER 1

INTRODUCTION

1.1 Background information

All living organisms are made up of cells. These cells, billions of which form our tissues and organs have what is called cell membrane (Rodamporn, 2010; Rodamporn et al., 2011). The cell membrane safeguards and surrounds the cell from outside environment. The cell membrane is semipermeable, that is, it permits some molecules and ions to pass through it and prevents others from entering the cell (Kotnik et al., 2012; Kotnik, Pucihar & Miklavcic, 2010). For a biologist, a cell is the building brick of life, containing a number of various organelles. Each organelle accomplishes a unique task and together they allow the cell to perform its function (Pucihar, Kotnik, & Miklavcic, 2009). On the other hand, from an electrical perspective, a cell is considered as a conductive cytoplasm enclosed by an insulating membrane and also surrounded by a conductive exterior or medium (Teissie et al., 2008).

The cell membrane is made up of a phospholipid bilayer with protein and ions embedded in it. Many of these ions in the cell membrane have a concentration gradient across the membrane (Rodamporn et al., 2011). For instance, potassium ion has a higher concentration in the intracellular region while sodium and chloride ions have a lower concentration in the intracellular region and vice versa in the extracellular region (Kotnik et al., 2010). These concentration gradients give rise to potential energy that resulted in the formation of membrane potential called resting membrane potential (Pucihar et al, 2008). The resting membrane potential value
ranges from -40 to -80mV, depending on cell type, size, and shape (Zupanic & Miklavcic, 2010; Pucihar et al., 2008).

When a cell is placed in an external electric field, a local charge redistribution occurs on both side of the membrane (Pucihar et al., 2009) due to the attraction of unlike charges and repulsion of like charges (positively charged ions are attracted to cathode electrode while negatively charge ions are attracted to anode). This charge redistribution induces a voltage on the membrane which superimposes on the resting membrane potential (Zupanic, Kos & Miklavcic, 2012). If the induced voltage reaches a threshold value usually between 0.2 to 1.5V, there is a localized structural rearrangement of the cell membrane, thus, micro holes are created in the membrane (Golberg & Rubinsky, 2009). Consequently, ions and molecules that are otherwise impermeable to the cell membrane become permeable to it. This process is called electroporation (EP) or electroporationization (Miklavcic & Towhidi, 2010; Neumann, 1992).

Depending on the electric field parameters (pulse strength, duration, number and repetition rate) exposing a living cell to an electric field may result in either permeabilization of the cell membrane, its destruction or has no effect (Kanduser, Sentjurc & Miklavcic, 2006). Therefore, the electric field parameters play a significant role in electroporation or Pulse Electric Field (PEF) method. If these parameters are within the threshold values (above the lower limit but below an upper limit), the consequence of the field application is the creation of transient pores which are used for introduction of molecules in or out of the cell (Weaver, 2000; Kanduser et al., 2006). In this case, the cell membrane can reseal few minutes after the withdrawal of electric field and this is termed as reversible electroporation (Zupanic, 2010) (refer to Figure 1.1). Meanwhile, if the parameters of the electric field are above an upper limit, the consequence of the field application is cell membrane destruction and this is termed, irreversible electroporation (Zupanic, 2010), as shown in Figure 1.1.

Ever since its discovery, electroporation has been a useful tool in many areas of medicine and biotechnology. At the moment, reversible electroporation is an established method for introducing chemotherapeutic drugs into tumour cells (Sersa et al., 2006; Kranjc et al., 2005; Snoj et al., 2005; Tozon et al., 2005). It also offers great promise as a technique for gene therapy without the risks caused by viral
vectors (Golzio, Rols and Teissie, 2004; Hojman, Gissel & Gehl, 2007). It is also commonly used in cell fusion (Orentas et al., 2001; Gaynor, Wells & Oback, 2005). In clinical medicine, irreversible electroporation is being investigated as a method for tissue ablation (Rubinsky, Onik & Mikus, 2007; Lee, Loh & Kee, 2007), whereas in biotechnology, it is useful for extraction of biomolecules and for microbial deactivation, particularly in food preservation (Rubinsky, 2007). Besides the effect of opening pores in the cell membrane, electroporation can change the cytoskeletal restructure that has an influence on the cell adhesion and migration (Titushkin & Cho, 2009). For example, redistribution of actin cytoskeletal has been shown in the cell electro-migration caused by direct current electric field (Weijer, 2009).

The interaction of living cell and biomaterials assumes a crucial role in tissue engineering and medical implant (Croll et al., 2006; Kladakis & Nerem, 2004; Liu et al., 2008; Nerem, 2006). A standout amongst the most well-known strategies proposed for tissue engineering includes; setting cells on a platform or scaffold, letting the cell to attach and proliferate in vitro, and subsequently embedding the cell construct into the patient (Martins, Reis, & Neves, 2008; Sefat, 2013). Therefore, it is

Figure 1.1: Cell exposed to an external electric field. (A) No effect, because the field is too small to cause permeabilization. (B) Cell reseal after electroporation. (C) The membrane is damaged due to high field intensity irreversible electroporation (Zupanic et al., 2012)
important to control the spreading and proliferation rate of the cells on the scaffold so as to optimise the cell culture environment, scaffold properties, and implant timing. The development of tissue on a scaffold comprises of four essential stages. These include cell attachment, cell proliferation, cell migration and cell differentiation on the scaffold (Khagani, 2012; Bradshaw et al., 2014). Similarly, wound healing is normally characterized by three processes; cell proliferation, cell migration and cell differentiation (Suzuki et al., 2003). The main cause of poor wound healing process is the deficiency in cell migration at the site of the injury (Fujii, Shearer & Azuma, 2015). Cell migration is also a significant course in the wound healing process besides the growth of an organism (Mackay, 2008; Xiao et al., 2011). Therefore, pulse electric field (Electroporation) could be used to facilitate the cell attachment, proliferation, adhesion and migration process which could further accelerate these cellular processes in tissue development.

Growth factors and extracellular matrix (ECM) protein such as laminin and fibronectin were found to increase the speed of re-epithelialization, therefore it plays a vital role in wound healing process and tissue regeneration (Metcalfe & Ferguson, 2007; Fujii et al., 2015). Laminin was found to enhance the keratinocytes cells migration (Choma et al., 2007; Seifert et al., 2012) while fibronectin was found to increase the adhesion and migration of epithelial cells (Kimura et al., 2006). Transforming growth factors beta (TGF-βs) are involved in signal transduction between the cell nucleus and extracellular environment (Raftery & Sutherland, 1999). TGF-βs are specialized molecules that are required in the regulation of cell adhesion, migration, proliferation, and apoptosis (Khaghani, 2012). Like many other growth factors, TGF-βs were thought to be stimulators of cellular proliferation (Morikawa, Derynck & Miyazono, 2016; Roberts et al., 1985). However, after several studies, TGF-βs were accepted to be bi-functional regulators that can either stimulate or inhibit the proliferation of many cell lines, depending on the circumstance and the environment (Morikawa et al., 2016).

Commonly, there are two types of electric waveforms used in electroporating cell membrane; exponential and square waveform (Puc et al., 2004; Markar, 2012). For an exponential waveform, a voltage chosen to be applied to the membrane will decay exponentially to zero with time, depending on the capacitance and resistance of the sample (Markar, 2012). When an exponential wave is used with high ionic
electroporation buffers such as serum and phosphate buffer saline (PBS), a high-value capacitance is required in order to electroporate mammalian cells (Morss, 2012; Markar, 2012). For a square waveform, a voltage chosen to be applied to the cell membrane remains constant for a specified period of time before returning to zero. The time duration of the voltage applied is called pulse length of the applied voltage (Markar, 2012). A square waveform is recommended for mammalian cells because it is softer and allows multiples pulses to be applied at a different repetition rate and it can be easily manipulated (Jordan et al., 2008).

1.2 Problem statement

The inducement process of pulse electric field for cells is still not totally comprehended (Valic et al., 2003; Chengxiang, Caixin & Chengu, 2011). Different applications of pulse electric field require distinct electric field parameters for different cells type (Pucihar et al., 2011). Thus, scrutinizing the electric field parameters for different applications and for a different type of cell is extremely critical, thus, comprehensive experimental information is needed (Chengxiang et al., 2011). In addition, there is proof that pulse electric field can extensively give a significant impact on the spreading and adhesion properties of numerous cells (Wang et al., 2003; Pehlivanova, Tsonova & Tzoneva, 2012).

The use of electroporation (PEF) together with the Chemotherapeutic drug (Electrochemotherapy) for cancer treatment has reached clinical trial (Sersa et al., 2000). However, patients undergoing electrochemotherapy in the clinical trial are complaining of unpleasant sensation due to muscular contraction during the pulse delivery (which is usually 1000V/cm for 100µs, 8 pulses at a repetition rate of 1Hz) (Mir & Orlovski, 1999; Lebar et al., 2002). This sensation is believed to be due to high amplitude of the pulse or due to the low repetition rate of the pulse. Therefore, there is a need to investigate for lower pulse amplitude that will result in the same efficiency of permeabilization and viability with the standard pulse parameter in order to potentially reduce the unpleasant sensation associated with the standard electric parameter used in electrochemotherapy.

Electric field was found to stimulate the secretion of growth factors and interact with ECM element to enhance cellular behaviour during wound healing
process (Mccaig et al., 2005). Cell assembly and alignment to guidance cues were found to play a key role in wound healing application (Jamil et al., 2007). Numerous researchers have examined cell alignment to the guidance cues via microcontact printing patterns of various ECM proteins (Jamil et al., 2007; Khaghani, 2012; Sefat, 2013). However, cell alignment to guidance cues in the presence of pulse electric field has not been investigated. Therefore, there is a need to look at the effect of pulse electric field which could further enhance cell alignment to the protein pattern surface for tissue engineering application.

1.3 Aim and objectives of the research

The overall aim of this study was optimization of low amplitude microsecond PEF on the cellular behaviour of epitheli um cell line for potential wound healing application. In addition, the effect of the PEF in the presence of extracellular matrix (ECM) proteins (laminin, fibronectin and collagen) on cell assembling to guidance cues and the cell behaviours in the presence of TGF-β3 would also be studied. The following are the objectives of the research:

1. To optimise the best PEF parameter for the growth of HT29 cells line.
2. To examine HT29 cell line responses such as attachment, proliferation, adhesion and migration under the influence of the optimum PEF parameter.
3. To establish a low field strength electric field that can give similar electroporation efficiency with the standard electric field parameter used in electrochemotherapy (SEFPECT).
4. To explore the effect of pulse electric field on the alignment of HT29 cells line on ECM proteins pattern via microcontact printing technique.
5. To demonstrate HT29 cell line behaviour such as proliferation and adhesion under the combined influence of optimum PEF and TGF-β3.
1.4 **Scopes of the study**

In order to achieve the objectives of this research, the following scopes of work were identified and followed.

1. HT29 cell lines were acquired and cultured in a physiological environment similar to that of the host tissue (mammalian colon). The cells were maintained at temperature of 37°C and humidified environment at 5% CO₂ in order to maintain the pH of the medium similar to pH of the human blood plasma.
2. Investigation of the best PEF parameter for the growth of HT29 cell line *in vitro*.
3. Real-time visualisation of the cellular morphological changes during cell attachment, migration and adhesion under the influence of PEF.
4. Exploration of a lower field strength that would potentially reduce the unpleasant sensation resulting from the used of 8 pulses at 1000V/cm, 100µs and 1Hz that is currently in use as SEFPECT.
5. Cell guidance of HT29 cell line on ECM protein pattern surface under the influence of PEF *in vitro* via microcontact printing technique.
6. Observation and examination of cellular behaviour in the presence of TGF-β3 and PEF.

1.5 **Thesis structure**

This thesis is structured into nine chapters as follows;

**Chapter 1:** In this chapter, a brief and general background on the research is provided. Additionally, the research problem formulation, aim, objectives and scope is also described in this chapter.

**Chapter 2:** This chapter gives a background and literature review on the state of the art of pulse electric field (PEF) or electroporation. In addition, a brief description of a cell and its electrical characteristic is given in this chapter.

**Chapter 3:** This chapter provides a list of general materials and equipment used in carrying out the research. A detail description of the general methods used in achieving the research goal is given in this chapter.
Chapter 4: In this chapter, the optimal electric field parameter for the growth of HT29 cell line is investigated. This optimal parameter is used in the subsequent chapters to study the influence of PEF on cellular behaviours.

Chapter 5: In this chapter, the influence of PEF on the cellular behaviours such as attachment, proliferation, spreading, adhesion and migration were studied. The cell attachment and migration were observed under time-lapse video microscopy.

Chapter 6: This chapter investigates a lower pulse strength that gives similar efficiency in terms of viability and permeability with standard electric field parameter used in electrochemotherapy. This will eliminate or reduce the unpleasant sensation experienced by patients during clinical electrochemotherapy.

Chapter 7: In this chapter, PEF effect on the HT29 cell assembly and guidance on the ECM protein micro-patterned surface, via microcontact printing technique were investigated.

Chapter 8: In this chapter, the combined influence of PEF and TGF-β3 on HT29 cell line proliferation, spreading, and adhesion was investigated.

Chapter 9: Finally, this chapter summarizes the conclusion of the results obtained in the study and gives some recommendations for future work.

1.6 Contributions of the study

In this research, the influence of PEF on HT29 cells line behaviour such as proliferation adhesion and migration, in the presence of ECM and transforming factor have been investigated. The following findings were revealed during the research

1. The optimum or best parameter for the growth of HT29 cell line at lower field strength has been identified to be at single pulse of 600V/cm with 500µs duration.

2. The research has revealed that PEF at the optimum value (at low field strength) has great influence on the cell attachment, proliferation, migration and adhesion which are the basic characteristics of wound healing and tissue regeneration. The PEF was found to increase the rate of cell attachment, proliferation, and migration and at the same time reducing the rate of cellular adhesion. This has
great implication in facilitating wound healing process and tissue regeneration when further investigated.

3. Additionally, the research has also found that an alternative field parameter of 600V/cm at 5ms could potentially reduce the unpleasant sensation associated with standard parameter currently used in electrochemotherapy. This parameter when further investigated could offer a new prospective in ECT in both pre-clinical and clinical trials.

4. Furthermore, the research demonstrated that the use of PEF in cell assembling and guidance to protein pattern via microcontact printing technique has further facilitated and enhanced the cellular alignment and elongation to guidance cue. This outcome potentially has great implication in directed cell migration for wound healing and tissue regeneration application.

5. Finally, the research has also demonstrated that combination of PEF and cytokines (TFG-β3) could be used in facilitating wound healing process and tissue regeneration by reducing the cell adhesion strength and increasing the cell proliferation rate.

In the next chapter, the review on current state of art on electroporation and its application on living cell for wound healing and tissue engineering is discussed.
CHAPTER 2

LITERATURE REVIEW

2.1 The cell

Cells are first discovered by Robert Hooke in 1665 from a slice of cork. Cells that are often called building blocks of life are the smallest functional and structural unit of all living things (Rodamporn, 2010). Cells emanate from the pre-existing cell through cell division. They have the ability to carry out all the activities of life. The cells are categorized into two groups, namely; prokaryotic and eukaryotic cell. Prokaryotic cells are smaller in size. They have a typical size between one to ten micrometres and they have no nucleus (Maskarinec, Wu & Lee, 2006). An example of a prokaryotic cell is bacteria cells. Figure 2.1 is a diagram of a prokaryotic cell.

On the other hand, eukaryotic cells are bigger in size and have a size between ten to hundred micrometres. They have a cell nucleus where they store genetic components like DNA and RNA. Their cell mass is around 1 nanogram. The examples of eukaryotic cells are animal, plant, fungi and Protista (Maskarinec et al., 2006). How a typical eukaryotic (animal cell) cell looks like is shown in Figure 2.2.
Figure 2.1: A typical diagram of a prokaryotic cell
(http://academic.venturacollege.edu/sflagan/protected/Ch4_structures.pdf, 2016)

Figure 2.2: A typical diagram of a eukaryotic cell (Animal cell)
(http://academic.venturacollege.edu/sflagan/protected/Ch4_structures.pdf, 2016)
2.1.1 HT29 cell line

The Colon cell line (HT29) emanates from a 44-year-old woman of the Caucasian race and was removed from a large intestine adenocarcinoma tissue (Forgue-Lafitte et al., 1989). The HT29 cell morphology is a typical of epithelial cell when cultured in vitro. In order to develop an in vitro model to study the effect of electroporation on cell proliferation and cell migration, the HT29 cell line was used.

HT29 cells were chosen because they have the capacity to be differentiated in vitro in response to changes in their extracellular environment. These cell lines are also used in the cytotoxicity assessment of the new biomaterials, as they are capable of preserving their phenotypic features for a long period time in a culture environment, hence these tests can be further reproducible (Luna et al., 2011). The HT29 cells have retained certain characteristics of the normal tissue, such as hormone receptors (Forgue-Lafitte et al., 1989). Table 2.1 gives the details characteristics of the HT29 cell line.

Table 2.1: HT29 properties

<table>
<thead>
<tr>
<th>Elements</th>
<th>Specifications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Organism</td>
<td>Human</td>
</tr>
<tr>
<td>Tissue</td>
<td>Colon</td>
</tr>
<tr>
<td>Culture properties</td>
<td>Adherent</td>
</tr>
<tr>
<td>Morphology</td>
<td>Epithelial</td>
</tr>
<tr>
<td>Gender</td>
<td>Female</td>
</tr>
<tr>
<td>Ethnicity</td>
<td>Caucasian</td>
</tr>
<tr>
<td>Age</td>
<td>44 years</td>
</tr>
</tbody>
</table>

2.1.2 The cell membrane

The cell membrane or plasma membrane is a living membrane that separates the inside of the cell from the outside surroundings (Zaharoff et al., 2008). The cell membrane is semipermeable to certain ions and organic molecules and is capable of regulating what enters and leaves the cell. It also facilitates the transport of materials that are necessary for survival. The membrane also maintains the cell electric
potential (Hille, 2001). Therefore, the cell membrane acts as a selective filter, which allows only some molecules to come out of the cell. Figure 2.3 shows a typical diagram of a cell membrane.

Virtually all animal cells are bounded by a cell membrane which is composed of a lipid bilayer with protein surrounds. The cell membrane also encloses the cytoplasm and has a typical thickness of 10nm (Maskarinec et al., 2006). Proteins constitute 30% of the cell membrane (Maskarinec et al., 2006). The lipid bilayer of the membrane is very delicate, and many forms of trauma can interrupt its function as the controller of molecules moving in and out of the membrane. The cell membrane also acts as a communication path between the cell and its extracellular environment via gap junction (Kelvin et al., 2000).

![Figure 2.3: A diagram of a cell membrane](http://www.vance-miller.net/2016/08/download-free-cell-membrane-coloring-sheet)

### 2.1.3 Membrane voltage

The variation in voltage between the internal and external surrounding of a living cell is called the membrane potential or voltage. The typical value of the membrane voltage range is from -40 to -80mV with respect to the exterior of the membrane (Kotnik et al., 2012). The membrane potential acts as a battery for supplying power to the various molecular devices in the membrane and also in transmitting a signal.
between different part of excitable cells such as neuron and muscles cells (Wang et al., 2010b).

Application of high intensity but a short electric field to a cell membrane increases the membrane potential. If the membrane potential value reaches a critical threshold value of 200mV to 1.5V, aqueous transient pores are formed in the cell membrane which can allow molecules that otherwise impermeable to the membrane to cross it (Neumann, 1992; Miklavcic, 2012). If field strength is not greater than the critical value (within the lower limit and upper limit for a particular cell), the membrane pores can reseal after some minutes. However, if the field strength is above the critical value of a particular cell, it leads to cell death or necrosis (Teissié et al., 2008; Edd & Davalos, 2007). The steady state induced membrane potential by an external electric field for a spherical cell is given by the Schwan equation as:

\[ V_m = 1.5RE \cos \theta \]  

(2.1)

Where \( E \) is the applied external electric field, \( R \) is the cell radius, \( \theta \) is the angle between the directions of the electric field and targeted point on the cell surface (Zupanic et al., 2012).

2.1.4 Cell cycle

Cells proliferation is necessary and important in all living organism for replacement of the dead cells and for tissue development (Hall, 2015). The cell proliferation is achieved within the context of cell division or cell cycle as shown in Figure 2.4 (Schmelzle & Hall, 2000). For a eukaryotic cell, the cell cycles comprise four distinct stages, namely; Mitotic phase or \( M \) phase, the synthesis or \( S \) phase, the gap 1 or \( G1 \) phase and the gap2 or \( G2 \) phase (Cooper & Hausman, 2000). The \( M \) phase is a part of the cell cycle whereby cell undergoes through mitosis and divides (Morgan, 2007; Cooper & Hausman, 2000). Following \( M \) phase, the cell enters into the \( G1 \) phase, which is a phase between mitosis and DNA duplication. At \( G1 \) phase, cells carry out the normal metabolic activity and continuous to grow but without DNA duplication (Brauer et al., 2008, Cooper & Hausman, 2000). From \( G1 \) the cell enters into \( S \) phase, whereby the duplication of DNA comes off. Following the duplication of DNA in \( S \) phase, the cell enters into the \( G2 \) phase. At \( G2 \) phase, the cell continues
to grow and synthesize protein in preparation for a new mitosis of another cycle (Morgan, 2007; Medema et al., 2000; Cooper & Hausman, 2000). At the G1 phase, the cell checks for any abnormality, like whether there is DNA damage and if there is, it checks whether it has been corrected. If the abnormality has not been corrected, the cell cycle is a halt at this phase (Tyson et al., 2002; Morris & Mathews, 1989).

A human cell in culture, which represents typical eukaryotic cell, divides in every 24 hours (Cooper & Hausman, 2000). For a rapidly proliferating human cell, the G1, S, G2 and M phases might last for 11, 8, 4 and 1 hours respectively (Cooper & Hausman, 2000). The evolution of cells via the division cycle is controlled by many external signals. This includes extracellular and intracellular signals that organize and monitor the processes that take place during the phase of the cell cycles (Gerhart, Wu & Kirschner, 1984; Hall, 2015).

### 2.1.5 Cell adhesion

The binding of cells together or to their environmental extracellular components is called cell adhesion. Cell adhesion involves protein molecules that extended across the cell membrane domain in both the intracellular and extracellular space (Hynes,
The cell adhesion protein in the extracellular region can either bind to the adhesion protein of adjacent cell (cell to cell adhesion) or bind to the extracellular matrix (ECM) protein (cell to ECM adhesion), (Sunami et al., 2006). The adhesion molecule usually binds to a molecule called its ligand. For instance, Cadherin and integrin are a ligand of adhesion protein between cell-to-cell and cell-to-ECM respectively (Farahani et al, 2014).

### 2.2 Extracellular matrix (ECM) protein

In addition to producing their own organelles, cells also produce the protein of the surrounding ECM (Hay, 2013; Alberts et al., 2002b). ECM is an interactive platform that regulates many cellular functions and also offers the cell anchorage and mechanical stability. Cells synthesise fibrous protein to produce their ECM proteins. The ECM perform three major functions namely: (a) provision of surface or substrate for cell adhesion and migration (b) it offers the strength and structural support for the cell and (c) it regulates and controls the cell metabolic function and cell differentiation (Ratner et al., 2004; Martini et al., 2001; Curtis & Riehle, 2001). The ECM has several components such as laminin, fibronectin and collagen (Ratner et al., 2004).

### 2.3 Cell signalling

Cell signalling is significant in ensuring cellular proliferation, migration, and differentiation (Kholodenko, 2006). ECM is molecule which is responsible for transmitting signal in and out the cells (Enderle, Blanchard & Bronzino, 2005). These signalings are categorized into two, the direct and soluble signals (Enderle et al., 2005). Direct signals are transmitted through an ion channels or receptors. The channels enable the diffusion ions in and out of the membrane for communication purposes (Martini et al., 2001). The soluble signal generation and transmission are further divided into autocrine, paracrine and endocrine. In autocrine, the signal is generated and acted upon by the same cell (Hay, 2013; Juliano & Haskill, 1993). In paracrine, the signal is generated by a cell and acted upon by neighbouring cells.
Whereas, in endocrine, the signal is generated by cells and acted upon by a distance cells (Alberts et al., 2002a).

2.4 Cell model

The general most acceptable model describing cell membrane is considering the membrane as spherical capacitor or spherical shell insulator (dielectric material) that separate the cell cytoplasm (cell interior) from the surrounding medium (cell exterior) (Escoffre et al., 2009; Zimmermann & Neil, 1996; Henslee et al., 2011; Weaver & Chizmadzhev 1996). The cell cytoplasm and exterior are considered to be good conductors of electricity. The membrane has a typical thickness of 6 nm (Coster, 2009).

When an external field is applied to the cell membrane, a transmembrane voltage is induced in the cell; most of the voltage is dropped on the cell membrane. When the induced voltage reaches a threshold value of 200mV to 1.5V (Zimmermann & Neil, 1996; Henslee et al., 2011), an electric field in the order $10^8$ V/cm (membrane voltage/membrane thickness = 1.5V/6nm) is created across the cell membrane. At this value of electric field strength, many dielectric materials will experience a breakdown (Coster, 2009). Hence, a re-structural rearrangement of the lipids bilayer occurs in the membrane as a result of charge redistribution, which leads to increase in the membrane permeability and conductivity (Golberg & Rubinsky, 2010a).

The transmembrane voltage can be computed using the Laplace equation under the quasi-static approximation with the assumption that both the membrane interior and exterior are good conductors. The induced transient transmembrane voltage $V_m$ is given by equation 2.2 (Escoffre et al., 2009).

$$V_m = 1.5RE\cos\theta fg \left(1-e^{-t/\tau}\right)$$

Where $f$ is a shape factor which has a value of 1 for a sphere, $R$ is the radius of the cell in meter; $\tau$ is the membrane charging constantly. $E$ is the applied electric field intensity; $\theta$ is the angle between the electric field and the direction of normal to the membrane; $g$ is a correction factor for a membrane that is not a perfect insulator. The
value of $g$ is given by equation 2.3 (Escoffre et al., 2009; Zimmermann & Neil, 1996).

$$g = \frac{1}{1 + G_m r + (0.5 \rho_i + \rho_e)}$$

(2.3)

Where $G_m$ is the membrane conductance per unit area and $\rho_i$ and $\rho_e$ are respectively the resistivity of the interior and exterior of the cell. Since in most cases the membrane conductivity is very small, $g$ can be approximated to be 1. Therefore, for a spherical cell ($f=1$), the value of induced transmembrane potential become

$$V_m = 1.5 r E \cos \theta \left( 1 - e^{-\frac{t}{\tau}} \right)$$

(2.4)

Equation 2.4 is called transient transmembrane potential. At a time much higher than the membrane charging time, the exponential term becomes zero. Therefore, equation 2.4 becomes;

$$V_m = 1.5 r E \cos \theta$$

(2.5)

Equation 2.5 is called the Schwan equation. From equation 2.5, it can be seen that the induced potential is directly proportional to the radius of the cell and angle between the electric field and direction of the normal to the membrane. Note that once the membrane permeabilizes, equation 2.5 is no longer valid because of the membrane dielectric breakdown. In other words, the membrane ceases to be an insulator (Morss, 2012). After permeabilization, increasing $E$ affects the increase in the value of transmembrane potential. However, the membrane conductivity significantly increases due to the flow of ionic current through the membrane (Morss, 2012; Zimmermann & Neil, 1996).

### 2.5 Theory of electroporation

A phenomenon in which a cell is exposed to a short but high-voltage electric field that momentarily disrupts the lipid bilayer and proteins of the cell membrane is called electroporation or electropermeabilization (Zimmermann & Vienken, 1982). There are protein channels, pores, and pumps present in the cell membrane. The closing and the opening of many of these channels constituted by proteins being
dependent on transmembrane potential (Kotnik et al., 2012). When an electric field is applied, many voltage sensitive protein channels will open. Once open, the protein channels will experience a current much greater than the current usually experienced by the protein channels during metabolic activities.

Consequently, the protein channels are irreversibly denatured (the disruption of the protein molecule from its three-dimensional shape that eliminates its enzyme activities is denaturation) by Joule heating or electric modification of functional groups such as hydroxyl, carboxyl, sulfhydryl, or amino group (Suzuki et al., 2011; Kotnik et al., 2010). The lipid bilayer is also vulnerable to the applied electric fields due to its net electric charge (Tieleman, 2004). The use of an electric field causes lipid molecules to reorient, thus creating hydrophilic pores. Hydrophilic pores conduct current, thereby generating local Joule heating and bring thermal transitions of the lipid bilayer from a firm gel structure to a liquid crystalline structure. Therefore, electroporation in the cell membrane occurs both in the protein channels and the lipid bilayer (Ho & Mittal, 1996; Tsong, 1991).

2.5.1 Stages of electroporation

The electroporation method comprises different stages. The first of them is pore creation, which is the cell membrane’s reaction to the induced transmembrane potential and survives for few microseconds (Kanduser & Miklavcic, 2009). The second stage is a time dependent enlargement of the pore size happening in a time range of hundreds of microseconds to milliseconds and lasts throughout the duration of pulses. The last phase is membrane recovery, which starts immediately after the removal applied electric pulse and consists of pore resealing, and survives for few minutes (Leontiadou, Mark & Marrink, 2004). The first stage of electroporation can be measured by changes in membrane conductivity and is related to short-lived transient pore formation, which does not contribute to molecular transport (Pavlin, Leben & Miklavcic, 2007). Molecular transport through the permeabilized cell membrane related with electroporation is detected from the pore formation stage until membrane resealing is completed (Puc et al., 2003; Pavlin et al., 2007).
2.5.2 Parameters affecting electroporation

Factors such as electric field parameters, the chemical composition of media and cell characteristic can affect the efficiency of electroporation. The most influential among these factors is electric field parameters. The most important electric pulse parameters are amplitude, duration, number and repetition frequency (Lebar & Miklavcic 2001; Canatella et al., 2001). If those parameters exceed the optimal values, irreversible electroporation takes place due to cell membrane disintegration resulting in cell lysis (Canatella et al. 2001). The choice of electric pulse parameters thus depends on the desired application. Some applications require reversible, while others require irreversible electroporation. For loading of foreign molecules into the cell, reversible electroporation is required.

The choice of electric pulse parameters depends on the type of the foreign molecule that is being introduced. For small molecules, such as different drugs or fluorescence dyes, a train of relatively short pulses (time duration in the range of microseconds to milliseconds) is sufficient. For large molecules, such as DNA, longer pulses (range of few milliseconds) or a combination of high-voltage short-duration pulses and low-voltage long-duration pulses is used (Satkauskas et al., 2002).

Besides the aforementioned parameters of electric pulses, different pulse shapes can also be used. The most frequently used are exponential and square wave pulses. Electric pulses can be applied in one direction or their orientations can be changed during the pulse application. Such protocols were successfully used for electrochemotherapy and gene electrotransfer (Golzio et al., 2004; Faurie et al., 2005; Rebersek et al., 2007).

2.5.3 Application of electroporation

2.5.3.1 Electrofusion

The use of electrical pulses can result in membrane fusion of neighbouring cells in close contact when suitable physical conditions were achieved (Jordan, Neumann & Sower, 2013; Zimmermann, 1986). Electrofusion leads encapsulation of both
original cells intracellular membrane material and can be used to obtain genetic hybrids cell or hybridomas (Talele et al., 2010). Hybridomas are hybrid cells created by the fusion of an antibody discharging stimulated B-lymphocytes, with a tumour cell that develops well in culture (Orentas et al., 2001). Electrofusion approach was successful in the reconstructed embryo in mammalian cloning (Gaynor et al., 2005).

### 2.5.3.2 Electrochemotherapy

The use of chemotherapeutic drugs combined together with electroporation is called electrochemotherapy (ECT). ECT facilitates the delivery of chemotherapeutic drugs to the malignant cell (Pavšelj & Miklavčič, 2008). Many chemotherapeutic drugs cannot cross the cell membrane under normal condition. Therefore, with the help of electrochemotherapy, this can be easily achieved by creating pores in the cell membrane by the use of an electric field (Pavšelj & Miklavčič, 2008). Commonly used drugs for chemotherapy such as bleomycin and cisplatin were found to be much more effective in the electrochemotherapy than in only chemotherapy when applied to a tumour cell lines both in vitro and in vivo (Mir et al., 2006; Sersa, Cemazar & Snoj, 2009).

### 2.5.3.3 Electrogenetherapy

The process of transferring DNA into cells to affect some form of gene therapy is often referred to as electrogenetherapy. Electrogenetherapy is currently being applied in some clinical trials (Heller & Heller, 2006). Additionally, it is also currently being considered to having a large potential as a non-viral method of delivering genetic material into cells, a technique meant for correcting genetic diseases (Heller & Heller, 2006). The genes being coiled up need a larger electropores for a longer time in order for it to enter the cells. Numerical modelling is useful to establish appropriate parameters to achieve this (Talele et al., 2010; Zhao, Zhang, & Yang, 2010). A successful gene transfer process is the one where the electrical and living conditions of the cell are such that the barrier function of the cell membrane is rapidly restored for a cell survival. This process is termed electogene transfer and when used for therapeutic purpose, electogene therapy.
2.5.3.4 Electroinsertion

Another use of electroporation is the insertion of molecules or ion into the cell membrane. As the pores created by the electric field in the membrane reseal, they entrapped some of the transported molecules (Talele & Gaynor, 2008). Experiments on Electroinsertion advocate the likelihood of applying the method to study certain physiological properties of the cells and understanding features of the lipid-protein interactions of the cell plasma membrane (Talele & Gaynor, 2008).

2.5.3.5 Electro-sterilisation

Pulse electric field is used as a promising method in the cold sterilisation of liquid food and drug. In this process, an irreversible electroporation is used to permanently destroyed the micro-organisms in the food or drug without changing its temperature, which may be the case when the thermal method was employed (Jaeger, Balasa & Knorr, 2009).

2.6 Pulse electric field effect and cell injury

An electric field ($E$) is defined as the ratio of the potential difference between two points ($V$) and the separation distance ($d$) between them, that is $E = V/d$. An electric field is a derived quantity measured in volt per centimetre ($V/cm$) or volts per meter ($V/m$). An electric field in a conductive medium generally produces current and vice versa (Song et al., 2007; Zhao, 2009). In a living organism, there are two predominant direct currents (DC) voltage sources namely; the transmembrane voltage in the cell membrane and trans-epithelial voltage that is present across a cell sheet interconnected through a high resistance tight junction (Messerli & Graham, 2011). An injury to this junction will create a low resistance pathway at the injury site. Therefore, due to the presence of the transmembrane and trans-epithelial voltage, coupled with the low resistance path (conductive path) created as a result of the injury, a current would flow at the injury site. Consequently, cells at the site of the injury are directed to migrate in the direction of the current to close the wound (Zhao, 2009; Messerli & Graham, 2011). Hence, it has been a question that
application of external field could induce additional voltage on the cell membrane which may further facilitate cell repair, by increasing the current flow provided it is not high enough to cause cell damage (Nuccitelli, 2003; Zhao et al., 2006; Messerli & Graham, 2011).

2.7 Electric current correlation with body sensation

Electric shock is defined as a physiological response, injury, or sensation triggered by electric current passing through our body (Lipman, 2007). It happens when our body comes in contact with any source of electricity that can cause a sufficient current to pass through the skin. The least current a human can sense depends on the current type (Alternating current (AC) or Direct Current (DC)) as well as frequency for AC. A person can feel at least 1mA (rms) of AC at 60 Hz, while at least 5 mA for DC (Clifford, 2005; John, 2005). If the current is high enough, it can cause tissue damage or fibrillation which can result to cardiac arrest. An AC Current of 30mA or DC current of 300–500mA can cause fibrillation (Clifford, 2005; Hobbs, Ossenkop & Lathan, 2017). The rule of thumb is 50V for AC or 120V for DC is considered the hazard limit (Hobbs et al., 2017; John, 2005). However, the voltage level can change depending on the humidity and body conditions (wet or dry). Therefore, to say whether the voltage is unsafe depends on the condition of the body (because body resistance varies with the condition of the body either wet or dry).

2.8 Cell viability

Cell viability measurement is used to determine the number of living cells in a total sample (Strober, 2001). The test for cell viability usually involves looking at the sample of the cell population and then staining the cells with trypan blue. The sample is then subjected to microscopic examination to evaluate which cells are viable (Wang et al., 2010a). A viable cell with intact membrane will exclude the dye and appear very bright under bright field microscopy. Whereas, a dead cell with porous membrane will absorb the dye and appear very dark under bright field microscopy (Wang et al., 2010a; Strober, 2001).
2.9 Microcontact printing technique

Micro-contact printing (MCP) is a scheme that allows a substrate to be functionalized freely with ECM protein, in a well-defined array (Ricoult et al., 2012; Berends, 2012; Khaghani, 2012). MCP also provides room for regulation of cell adhesion geometry on a substrate (Sefat, 2013). Microcontact printed surface may be used to study cellular behaviour with respect to a wide range of ECM protein such as laminin, fibronectin, and collagen (Park & Shuler, 2003; Khaghani et al., 2008). Laminin, fibronectin, and collagen are the principle component of extracellular matrix protein and perfect molecule for micro-contact printing (Sefat, 2013). This is because they can coordinate cellular behaviour like cell attachment, motility, development and differentiation in vitro (Han et al., 2007). This cellular behaviour could then be utilized to help in understanding the cell signalling pathways that are connected with the regulation of cell interaction with the surface (Stevens & George, 2005; Pawson & Nash, 2003).

2.10 Extracellular matrix protein as a tool for microcontact printing technique

ECM protein such as laminin (Lauer, Klein & Offenhausser, 2001), fibronectin and collagen (Yamamota et al., 2006), have been widely used to facilitate cell responses to guidance cues. In a nutshell, the creation of this ECM protein with a repeated stripe networks excites cell alignment and directed cell migration (Jamil et al. 2007). Nonetheless, various cells react to guidance cues of different sizes in different ways (Jamil et al. 2007). Extracellular matrix (ECM) protein is the main regulators of many cellular functions such as cell to cell adhesion, cell to ECM adhesion, cell communication and cell division (Raab et al., 2010).

Additionally, ECM also controls cell migration and cell shape. Understanding the process that controls cell function such as proliferation adhesion and migration is very significant for wound healing and tissue engineering application and in the development of new tissue in vivo (Shin, Jo & Mikos, 2003; Metcalfe & Ferguson, 2007). Laminin, fibronectin, and collagen are the major components of ECM protein. Each of this protein bind to a specific integrin
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