Microfluidics based electroporation for inactivation of microorganisms

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Microfluidics Based Electroporation for Inactivation of Microorganisms

**NANYANG
TECHNOLOGICAL
UNIVERSITY**

SINGAPORE

PUDASAINI SANAM

SCHOOL OF MECHANICAL AND AEROSPACE ENGINEERING

NANYANG TECHNOLOGICAL UNIVERSITY

2020

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Microfluidics Based Electroporation for Inactivation of Microorganisms

PUDASAINI SANAM

Supervisor:

Professor Yang Chun

**A thesis submitted to the Nanyang Technological
University in partial fulfilment of the requirements for
the degree of Doctor of Philosophy**

2020

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Statement of Originality

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Authorship Attribution Statement

This thesis contains material from papers published in the following peer-reviewed journals in which I am listed as a first author.

Chapter 3 is published as Pudasaini, S., Perera, A. T., Das, D., Ng, S. H., Yang, C., 2019. Continuous flow microfluidic cell inactivation with use of insulating micropillars for multiple electroporation zones, Electrophoresis. doi:10.1002/elps.201900150.

The contributions of the co-authors are as follows:

- I designed the experiment, fabricated the device, built experimental setup, and performed all the experiments. Additionally, I analyzed the data and prepared the manuscript drafts.
- Das, D. assisted during device fabrication and Perera, A. T. aided during data analysis.
- The manuscript was revised by Perera, A. T., Das, D., Ng, S. H. and Yang, C.

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The contributions of the co-authors are as follows:

- I performed all the device design, device fabrication, device operation and electrical measurement and conducted most of the analysis of results.
- Perera, A. T., Ahmed S. S. U. aided during the experiments as well as in preparing the sample.

- I prepared the manuscript draft. The manuscript was revised by all authors.

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The contributions of the co-authors are as follows:

- I designed the experiment, conducted all the device fabrication and performed the experiments.
- Chong, Y. B. aided in preparation and handling of the bacteria sample.
- Perera, A. T., Ahmed S. S. U. assisted during the experiments and data analysis.
- I prepared the manuscript drafts. The manuscript was revised by all authors.

January 19, 2020

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Date

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Pudasaini Sanam

Abstract

Disinfection of water has prevented humans from deadly water-borne diseases for many years. Current methods of bacterial inactivation include the use of ultraviolet radiation (UV), chlorine compounds, ozonation, etc., which pose issues such as high energy consumption, the formation of disinfection by-products, pathogen regrowth, etc. This PhD thesis reports electroporation-based inactivation devices that achieve highly efficient bacterial inactivation at significantly lower voltage compared to the conventional bulk electroporation device. The method involves the use of insulating structures such as micropillars and microbeads that create multiple electroporation zones with enhanced local electric field strengths enabling bacterial electroporation at low voltages.

Firstly, as a proof of concept, a simple microfluidic electroporation device that includes an array of micropillars was developed. Nearly 63% of inactivation of *Saccharomyces cerevisiae* was achieved at a flowrate of 2.5 mL/h, which was higher than the 24% cell inactivation observed for a micropillar-free reference device subjected to the same conditions. Further, to investigate the effect of micropillar shapes on the inactivation performance, two microfluidic devices with different base geometries (circular and rhombus) were developed. The effect of DC and AC voltages were also examined along with other parameters, including applied frequency, and flowrate. The parametric experimental studies showed a higher log removal efficiency for the rhombus-shaped micropillar device than the circular micropillar device, where *Escherichia coli* and *Enterococcus faecalis* were used as two model bacteria. Scanning electron microscopy (SEM) imaging was performed confirming the membrane damage was caused by electroporation.

Secondly, an electroporation-based bacterial inactivation device operating in an insulating microbeads-enhanced electric field was developed. Electric voltage was applied to a pair of mesh electrodes inside the chamber consisting of densely packed microbeads. The microbeads generate locally high electric field strengths at low applied voltages, which were sufficient for the electroporation of bacteria. The effect of microbeads on the inactivation performance was assessed by comparing the performance of the microbead device with that of a microbead-free device under the same operating conditions. The results showed that the microbead-free device achieved only 0.57 log removal—eightfold lower than that for the device with microbeads. The energy consumption of the device was much lower than that of the UV inactivation of bacteria.

Finally, a contactless insulator-based dielectrophoresis device was developed to enable bacterial trapping in the region of high electric field strength using positive DEP and thereby increasing the holding time of bacteria for a better electroporation performance. The device involved two side channels that were filled with liquid metal and were isolated from the main channel by a thin strip of polydimethylsiloxane (PDMS). The developed finite element electric field simulation showed good agreement with theoretical electrical circuit modeling in determining the voltage-drop across the medium. Significantly higher bacterial trapping and inactivation were achieved at significantly lower voltages compared to the conventional bulk electroporation devices.

Overall, the reported method in this PhD work could potentially be used in different applications of electroporation such as water treatment, food processing and continuous extraction of intracellular components.

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DEDICATED

To

My wife, Sukirti Dhakal

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List of Acronyms

2D	2-dimensional
3D	3-dimensional
AC	Alternating current
BEP	Bulk electroporation
BSA	Bovine serum albumin
CNT	Carbon nanotubes
CuONW	Copper oxide nanowire
DEP	Dielectrophoresis
DC	Direct current
DI	Deionised
DNA	Deoxyribonucleic acid
<i>E. coli</i>	<i>Escherichia coli</i>
EDL	Electric double layer
<i>E. faecalis</i>	<i>Enterococcus faecalis</i>
EP	Electrophoresis
EO	Electroosmosis
EOF	Electroosmotic flow
GFP	Green fluorescent protein
iDEP	Insulator-based DEP
kHz	kilohertz
IPA	Isopropyl alcohol
ITO	Indium tin oxide

LOC	Lab on a chip
MEMS	Micro electro-mechanical system
MHz	Megahertz
nDEP	Negative DEP
PBS	Phosphate buffered saline
pDEP	Positive DEP
PDMS	Polydimethylsiloxane
PI	Propidium iodide
PMMA	Polymethyl methacrylate
RNA	Ribonucleic acid
SCEP	Single cell electroporation
SEM	Scanning electron microscope
RBC	Red blood cell
TMP	Transmembrane potential
UV	Ultraviolet radiation
YPD	Yeast extract peptone dextrose

List of Symbols

U = Voltage

I = Current

E = Electric field

Subscripts

- 0 – Applied electric field

r = Radius of cell

d_m = Thickness of cell membrane

ϵ^* = Complex permittivity

Subscripts

- m – medium
- p - particle
- i – intracellular
- e – extracellular

σ = Conductivity (Subscripts are similar to complex permittivity)

ϵ = Permittivity (Subscripts are similar to complex permittivity)

φ = Electric potential

ω = Angular velocity

$\Delta\Psi_E$ = Transmembrane potential

τ = Membrane charging time

l = Channel width

w = Channel width

h = Channel height

u = Velocity

Subscripts

- eo – electroosmosis
- ep - electrophoresis
- p – particle

μ = Mobility (Subscripts are similar to velocity)

f = Frequency

ζ = Zeta potential

ρ_e = Net charge density

κ = Debye length

Re = Reynold number

Q = Volumetric flow rate

A = Cross sectional area

ϵ_0 = Permittivity of free space

Chapter 1: Introduction

1.1 Background

Globally, more than 2 billion people use drinking water sources that are contaminated with faeces (Peter and Umar 2018). According to the WHO, diarrheal illness caused by waterborne pathogens is estimated to cause over 485,000 deaths each year, and therefore treatment of contaminated water is of critical importance (WHO 2019). Moreover, due to the increasing world population and globally improved standards of living, water demand worldwide is constantly under pressure and therefore water treatment has special relevance. Modern water treatment processes have had a significant impact on waterborne diseases, and these processes constitute barriers between the consumer and such diseases. The aim of drinking-water treatment is to produce clean water that is chemically, biologically, and aesthetically safe. Depending on the technology of the plant and the quality of raw water, the water treatment process may vary at different locations, but the basic methods are largely the same.

The most common treatment process includes coagulation, flocculation, sedimentation, filtration, and disinfection as shown in Figure 1.1. Initially, raw water is collected at the reservoir, and then undergoes coagulation, where liquid aluminum sulphate (alum) and/or polymer is added. Upon mixing, these coagulants cause tiny particles to adhere together, i.e. coagulate. These tiny particles then adhere to others, forming larger particles called flocs. These flocs can be removed easily by filtration or sedimentation. Heavier floc particles, also called sludge, are collected at the bottom of the sedimentation basin and are removed. However, coagulation and flocculation cannot remove tiny particles and microorganisms. These tiny particles are removed using mechanical methods such as filtration, hydrocyclone, etc. Filtration is mostly used for

separating organisms as well as suspended solid materials. The suspended solids and waste resulting from filtration are either discharged in the same area from where they are loaded or further treated before discharging. Traditional granular media filtration is effective for particles greater than 50 μm (Gonçalves and Gagnon 2012). However, this allows the passage of smaller particles as well as microorganisms such as *Escherichia coli*. Moreover, the low filtration rate and large space requirements are also its limitations (Tang et al. 2006, 2009). Subsequently, crumb rubber filtration was introduced to increase filtration efficiency. This technology uses crumb rubber, which is produced from waste tyres, as a filtration medium. Filter beds are packed with rubber particles of various sizes (0.66, 1.2 and 1.9 mm). This method has relatively low density and requires less space, and the filter can provide a high filtration rate with a longer run time. However, this method is not preferred because of the compression of the crumb rubber medium during operation, which reduces its porosity (Gonçalves and Gagnon 2012). Another separation method, cyclonic separation, is also used for separating sediments and particles (Waite et al. 2003). This technology uses hydrocyclones and requires less pump pressure and is generally used as a pre-treatment process.

Figure 1.1 Typical water treatment processes

After filtration, pathogens are inactivated using various physical methods that include ultraviolet (UV) radiation (Wu et al. 2011), thermal treatment (Mesbahi et al. 2007), deoxygenation (McCollin et al. 2007), electrolysis (Dang et al. 2003) and strong electric field discharge-based OH radicals (Zhou et al. 2003). UV radiation is the most commonly used disinfection technology in water treatment. It causes a photochemical reaction with biological components of the microorganism. However, it has no effect on physical components such as sediment and hence requires a pre-treatment process. The effectiveness of UV radiation is also reduced in presence of huge volumes of algae (Bai et al. 2012). Thermal treatment involves heating large volumes of water over time (Quilez-Badia et al. 2008). Low-temperature thermal treatment typically requires 30–80 hr thermal treatment at less than $< 45^{\circ}\text{C}$. Consequently, this treatment is not feasible if continuous flow treatment is required. Corrosion of tanks and other piping components is also an issue when dealing with hot water for a prolonged period. On the other hand,

high-temperature thermal treatment overcomes this issue. Treatment is carried out at high temperatures (40–80°C) over a short time. However, the risk of operating high-temperature water is high, as it involves safety issues. Deoxygenation involves the asphyxiation of microorganisms using inert gases (McCollin et al. 2007). In this process, the propensity for corrosion is reduced significantly but the time required is generally 1–4 days. Moreover, this technology also requires highly sealed treatment tanks. Electrolytic treatment methods are also used in the treatment process. However, energy consumption is very high. Hydrogen gas and sodium hypochlorite (NaClO) are produced in this process, which is undesirable (Matousek et al. 2006), as hydrogen gas (16% v/v) increases the risk of explosion, whereas residual sodium hypochlorite and other by-products have adverse effects on human health.

Another commonly used disinfection method is chemical disinfection. The use of chemical substances can have other multiple environmental, health and safety impacts. Hence, additional approval processes may be required for this type of treatment (Resolution 2008). Oxidizing as well as non-oxidizing biocides are used as chemical substances to inactivate organisms (Kazumi 2007). Oxidizing biocides are general disinfectants that destroy the organic structure of microorganisms such as the cell membrane or nucleic acids. Chlorine, iodine and bromine are oxidizing biocides. Hypochlorous acid is the main disinfectant used in chlorine-based treatment. Hydrogen peroxide (H₂O₂) (Kuzirian et al. 2001) and ozone (O₃) (Oemcke and Van Leeuwen 2005) are other widely used oxidizing agents. However, before the water can be supplied, the residual chlorine needs to be neutralized. Moreover, due to the presence of a large amount of organic matter, a high dose of biocides is required for complete disinfection and thus increases the total residual oxidants. Similarly, non-oxidizing biocides interfere with metabolic, reproductive and neural functions. Glutaraldehydes

(Sano et al. 2005), seaKleen (Raikow et al. 2006) and PERACLEAN Ocean (De Lafontaine et al. 2008) are commercially available non-oxidizing biocides used for water treatment. The use of chemical biocides has been proven to be an efficient method for inactivating organisms. However, the use of chemicals presents a risk to the operator.

In a typical water treatment plant, two or more methods are combined for effective operations as well as efficient inactivation of organisms. Mechanical methods are generally used as a pre-treatment process. The main idea is to remove larger particles using separation techniques, followed by the inactivation of micro-sized organisms. Either physical or chemical methods are used as the main treatment process. UV inactivation combined with filtration is widely used (Kong et al. 2007; Sutherland et al. 2003). Ultrasonic treatment, as well as hydrogen peroxide, has also been combined with UV treatment (Sassi et al. 2005).

Although the common treatment methods can inactivate pathogens, they suffer several limitations. Chlorination produces carcinogenic by-products such as *N*-nitrosodimethylamine and trihalomethanes (Lee et al. 2007; Mitch et al. 2003; Tang et al. 2014). The use of UV inactivation is limited because of its high operation and maintenance costs, high energy consumption and dependence on the turbidity of the raw water (Guo et al. 2009; Guo et al. 2012; Krasner 2009). Similarly, ozonation requires an extremely high electric field to generate ozone and is costly (Gonçalves and Gagnon 2012; Tsolaki and Diamadopoulos 2010). Several electroporation-based inactivation devices have also been reported in the literature (Huo et al. 2017a; Huo et al. 2016a; Huo et al. 2018b). There is no by-product formation and cell reactivation is prevented, thus making this method a potential substitute for the traditional inactivation methods (Huo et al. 2016a). However, very high voltage is required to generate a

sufficiently strong electric field, resulting in high power consumption as well as posing operational safety risks. Electroporation-based treatment methods can be a potential substitute for the current treatment methods, however, the enhancement of electric field is necessary, as traditional pulsed electric field method involves the use of extremely high voltage to inactivate microorganisms, which is the motivation for the present study.

1.2 Scope of work

The main objective of this PhD work is to develop an electroporation-based inactivation of microorganisms that uses locally enhanced electric field and to further develop the fundamental framework for understanding the parameters affecting the process. This research work aims to utilize the insulating structures within microfluidic devices to demonstrate the electric field enhancement required to inactivate cells from a water sample.

The following research scopes are identified to fulfill the research objectives:

- Develop a micropillar-based microfluidic device to investigate the electroporation-based inactivation using yeast cells. Experimentally determine the appropriate electric field and flowrate associated with the inactivation process. Numerically investigate the electric field enhancement and field strength required for electroporation.
- Experimentally investigate the effects of micropillar geometry, applied electric field type, frequency, and the flowrate on two different bacteria types. Numerically explain the electric field enhancement associated with different micropillar shapes.

- Develop an electroporation-based inactivation device using silica beads and investigate the effect of electric parameters and the flowrate on bacterial inactivation. Develop a unit cell model and numerically investigate electric field enhancement due to the use of insulating microbeads.
- Demonstrate positive dielectrophoresis (pDEP) trapping of microorganisms for inactivation using a contactless insulator-based DEP device (iDEP). Further, experimentally investigate the role of various parameters affecting the performance of the device. Develop an electrical model to determine voltage-drop across the medium.

1.3 Outline of the thesis

This thesis consists of seven chapters including this introductory chapter, which introduces the background and motivation of our work.

Chapter 2 will present a review of the relevant literature. The chapter starts with a basic introduction of electroporation phenomena and its types. This is followed by a description of transmembrane potential (TMP), where the structure of the cell membrane is discussed and a short derivation of TMP is presented. Then, two popular theories of electroporation are discussed, namely the electromechanical compression theory and the aqueous pore model. Further, conventional bulk electroporation (BEP) and single-cell electroporation (SCEP) devices are detailed, where microfluidic SCEP devices are elaborated. The electrokinetic phenomena in an iDEP device are also discussed, and lastly, electroporation-based devices developed for application in water treatment are presented.

Chapter 3 will report the continuous-flow microfluidic cell inactivation using insulating micropillars for multiple electroporation zones where a systematic study on the effect of insulating structure, electric field and flowrate on cell inactivation was

investigated. This chapter includes a detailed fundamental study, device design and fabrication, and numerical and experimental methods for characterizing the inactivation performance of the device.

Chapter 4 will present the key findings associated with the bacterial inactivation in the two different microfluidic devices using different shapes of pillars. Here, the fundamentals of electroporation-based cell inactivation in the microfluidic device are briefly presented, followed by the experimental results associated with the parametric study, including electric field strength, electric field type (AC or DC), frequency and flowrate on two different types of bacteria. The numerical results showing and comparing the electric field distribution for the different geometric shapes used in the experiments are also covered.

Chapter 5 will report the use of insulating microbeads for enhancing the electric field to inactivate bacteria in a water sample. The chapter also demonstrates bacteria inactivation under different electric fields and flowrate conditions. Numerical results using a unit cell model for obtaining electric field distribution are also presented.

Chapter 6 will present the experimental and numerical studies on the use of the iDEP device involving contactless electrodes for simultaneous trapping and inactivating bacteria. Here, bacterial trapping is achieved in the region of high electric field strength, where the bacteria are eventually inactivated. pDEP techniques are employed and the device characterization, for the same, is presented herein.

Lastly, **Chapter 7** will conclude this thesis and will present future directions for the use of insulator-based devices for inactivating microorganisms from water samples. Finally, the extension of the present work is briefly presented as future work.

Chapter 2: Literature review

This chapter presents the literature involving cell electroporation and provides some background knowledge of the cell membrane and its structure, origin of resting potential, transmembrane potential (TMP) along with different theories of electroporation. Additionally, this section discusses conventional as well as modern methods of electroporation. Lastly, several microfluidic devices developed for various electroporation-based applications are elaborated while focussing on their application in water treatment.

2.1 Background on electroporation

Electroporation is the most commonly used technique in molecular biology, clinical research and biomedical engineering for delivering exogenous components into cells (Chang et al. 2016b). In this process, upon the direct application of an electric field, TMP is induced. When the TMP reaches a threshold, nanopores are formed within the cell membrane, allowing the exchange of foreign materials (Gehl 2003b). Figure 2.1 presents schematic of the electroporation process.

Figure 2.1: Schematic representation of an electroporation process. The figure presents how TMP originates when a cell is placed in an external electric field, the formation of nanopores and the delivery of foreign particles such as drugs and genes

Electroporation was first introduced by Zimmermann et al. (1974), where a dielectric breakdown of the cell membrane of red blood cells (RBC) and *E. coli* B was demonstrated using a Coulter counter. In their device, a homogenous electric field was applied using a high-voltage capacitor between two flat platinum electrodes. The release of haemoglobin from RBC was observed, confirming their theory of dielectric breakdown. However, the process was irreversible, and the cells were found to be ruptured. Over time, an extensive literature has been developed for studying the electroporation method. The first successful reversible electroporation was achieved by Neumann and colleagues using a short-circuit electrophoresis (EP) apparatus, where successful gene transfer into murine cells was achieved (Neumann et al. 1982). Since then, many electroporators have been developed, operating on exponentially decaying pulses to transfer DNA molecules to bacterial cells. In the 1980s, studies with eukaryotic cells were reported for various applications such as gene manipulation in plant cells, in vivo and in vitro drug delivery, antibody production as well as embryo cloning (Lee and Deng 2012). However, most devices involved the use of very high voltage (>1000 V), which resulted in high cellular mortality rates. The devices also suffered from unwanted joule heating effects, playing an important role in cell lysis.

Sale and Hamilton studied the effects of the strong electric field on yeast and bacteria. In that study, applied field strength up to 25 kV/cm was applied as a series of DC pulses and concluded that cell death was due to neither the by-products of electrolysis nor joule heating, but solely due to electric field strength. Moreover, the group reported that yeast was more sensitive than bacteria in terms of the treatment (Sale and Hamilton 1967). Similarly, the effects of the electric field on bacteria and yeast cells were further studied by Hülshager and associates in 1983. The authors carried out comparative studies between gram-negative bacteria, gram-positive bacteria and yeast cells, describing the lethal effects of the electric field. Cells growing in logarithmic phase were found to be more sensitive than cells in the stationary phase. In addition, gram-negative bacteria were more sensitive to electric field treatment than gram-positive bacteria and yeast cells (Hülshager et al. 1983).

Sato and colleagues studied the formation of chemical species along with their effects on microorganisms using pulsed high-voltage discharge in water. It was concluded that the formation of hydrogen peroxides due to the formation of OH radicals had severe effects on yeast cells. Moreover, the logarithm of the survival ratio decreased along with increased H₂O₂ concentrations (Sato et al. 1996). Schoenbach and colleagues studied the effect of pulsed electric fields on biological cells. They explored the lethal effects of a very short electric field in the range of 0.1–100 kV/cm on bacteria and aquatic nuisance species. The authors showed that the electrical energy required to kill microorganisms decreases when pulse duration is reduced. However, for microorganisms, this tendency was reversed for pulses of <5 μ s (Schoenbach et al. 1997).

In search of non-thermal food processing techniques, Russell and group investigated the mechanisms of action of a pulsed electric field on food poisoning bacteria (in

2002). They used field strength up to 20 kV/cm, in which membrane permeabilization resulted in increased UV leakage and decreased ability to maintain the pH gradient (Russell et al. 2000). Further, Malik et al. (2001) studied electric field discharge in water purification. They explored electric field treatment as an ozone-synthesis method as well. Several chemically reactive species were produced during the process. They concluded that the application of pulsed electric field during ozonation might result in better dispersion of ozone in water and speedy conversion of ozone into free radicals, which would ultimately reduce the cost of ozonation (Malik et al. 2001). Using an AC high electric field, Uemura and Isobe developed a new apparatus in 2002 for inactivating *E. coli* in saline water. They supplied a continuous AC field of 20 kHz with the strength of 14 kV/cm to 0.1% saline water. The increase in temperature during the process also assisted in the inactivation (Uemura and Isobe 2002).

Although many researchers believed there are electroporation-induced pores in the cell membrane, Chang and Reese (1990) reported it for the first time on RBC using rapid-freezing electron microscopy. They found that the membrane remained intact and smooth before applying the electric field, whereas volcano-shaped pores were observed after the application of high electric field strength for 40 ms. The pore size was around 2 nm initially, which then increased rapidly to 20–120 nm. Based on their studies, the authors observed that the pores remained stable for a few seconds before resealing. Later, many existing studies examined the typical behaviour of pores during electroporation on mammalian cells (Table 2.1).

Table 2.1 Dynamic pore behavior during electroporation on mammalian cells (Lee and Deng 2012)

Time after voltage is applied	EP status	Estimated pore size (nm)
< 1 ms	pore formation	1– 20
20 ms	pore expansion	20–120
1–10 s	pore resealing	~ 1

2.1.1 Reversible electroporation

TMP linearly increases with the applied electric field, and when it reaches a critical value, i.e. 0.2–1 V, nanopores are formed within the cell membrane (Gehl 2003b; Santra and Tseng 2013b). These nanopores are transient and can reseal under normal conditions if the electric field is removed, and the process is called reversible electroporation (Geng and Lu 2013). This process is dependent on the electroporation conditions and the temperature, and resealing can take a longer time (even minutes) (Teissie et al. 2005). This process is widely used for delivering cargo such as DNA, drugs, protein and nanoparticles into cells or tissue (Andre and Mir 2004; Gothelf et al. 2003; Rols et al. 1998). Several other mechanisms such as diffusion, electrophoresis and electroosmosis play an important role during the delivery process (Weaver and Chizmadzhev 1996). Moreover, the process also depends on the size and charge of the molecules (Chen et al. 2006). Electrophoresis is a widely used mechanism in electroporation-based transfection. Typically, short high-voltage pulses are used for electroporating cells, whereas long low-voltage pulses are used for transporting the molecules (Geng and Lu 2013).

2.1.2 Irreversible electroporation

Alternatively, if the damage is too severe, the pores do not reseal, and the process is called irreversible electroporation (Geng and Lu 2013). For irreversible breakdown, the

cell membrane is permanently perforated, and the cell becomes permeable to the suspended medium. Although the exact mechanism is still unclear, it is believed that molecular transport across the cell membrane caused by extensive permeabilization and chemical stress results in the formation of permanent pores (Weaver and Chizmadzhev 1996). This process is typically used for lysing cells to extract intracellular components such as DNA, proteins, mitochondria and RNA (Ganeva et al. 2003; Vitzthum et al. 2000). Additionally, the process selectively inactivates cells as well as microorganisms in food products (Eppich et al. 2000; Jeyamkondan et al. 1999).

2.2 Transmembrane potential

2.2.1 The cell membrane and its structure

The cell membrane, also called the plasma membrane, is a semipermeable membrane that separates the internal components of the cell from the external environment. The primary function of the cell membrane is to protect the cell from the external environment and to allow the selective exchange of ions and organic molecules. It consists of a phospholipid bilayer with a hydrophilic phosphate head and hydrophobic fatty acid tail joined by a glycol molecule. Proteins, carbohydrate and cholesterol are also embedded within this bilayer.

Figure 2.2 Phospholipid bilayer structure of the cell membrane (Online open source image)

The construction of the cell membrane is shown in Figure 2.2, and the typical parameters for the cell membrane are presented in Table 2.2. It is seen that the conductivity of cell membrane is much lower than that cytoplasmic conductivity.

Table 2.2 Typical parameters of the cell membrane (Kotnik et al. 1997)

Symbol	Description	Value
C_{memb}	Membrane capacitance	10^{-2} F/m^2
d	Membrane thickness	5 nm
σ_{int}	Cytoplasmic conductivity	0.2 S/m
σ_{memb}	Membrane conductivity	$5 \times 10^{-7} \text{ S/m}$

2.2.2 Mathematical derivation of transmembrane potential

When the external electric field is applied across the cell membrane, the ions inside the cell move and accumulate as charge on the cell membrane surface. This accumulation of charge induces potential across the membrane, called transmembrane voltage. Whenever the voltage across the cell membrane exceeds a critical value, called transmembrane voltage, pores are formed within the membrane. The critical value, in which the cell membrane becomes permeable to the medium, corresponds to TMP of

approximately 1 V as compared to -70 mV under normal conditions (Chang et al. 2016b). Due to the difference in ionic concentration inside and outside of the cell, voltage is induced across the membrane. Appendix B discusses the origin of TMP under commonly encountered conditions.

Figure 2.3 Schematic of induced TMP when a cell placed in an external electric field

Figure 2.3 presents the schematic of induced TMP when a cell is placed in an external electric field. The conductivity of the cell membrane is several orders lower than that of the cytoplasm and external medium (Pucihar et al. 2001). Therefore, when the cell is exposed to an external electric field, the membrane acts as a capacitor and charges are induced across the membrane, giving rise to a potential, also called induced TMP. Under a DC electric field ‘ E ’, the induced TMP for a spherical cell is given by Schwan’s equation as per Tsong (1991):

$$\Delta\psi_E = fgrE\cos\theta(1 - e^{-t/\tau}) \quad 2.1$$

where R is the radius of the cell and θ is the angle between the line of electric field and the line joining the centre of the cell to the point of interest. The form factor f is given by:

$$f = l/(l - 0.67r) \quad 2.2$$

where l is the length of the cell. For spherical cells ($l = 2r$), so $f = 1.5$; for rod-shaped cells ($l \gg 2r$), $f = 1$. Similarly, g is a complex function of the intracellular (σ_i),

extracellular (σ_e) and membrane conductivities (σ_m), and the cell membrane thickness (d_m), which is written as:

$$g(r) = \sigma_i \sigma_e / [\sigma_m (\sigma_i + 2\sigma_e)(r/2d_m) + (\sigma_i - \sigma_m)(\sigma_e - \sigma_m)] \quad 2.3$$

In the TMP equation, τ is the membrane charging time, which can be further written as:

$$\tau = rC_m(\sigma_i + 2\sigma_e) / [2\sigma_i \sigma_e + r\sigma_m(\sigma_i + 2\sigma_e) / d_m] \quad 2.4$$

where C_m is the specific capacitance of the membrane. In the case where the conductivity of cell membrane is assumed to be pure dielectric, i.e. $\sigma_m = 0$, then:

$$\begin{aligned} g &= 1 \\ \tau &= rC_m(\sigma_i + 2\sigma_e) / (2\sigma_i \sigma_e) \end{aligned} \quad 2.5$$

Moreover, for a spherical cell, if the charging time is much shorter compared to the DC pulse width or half time period of AC voltage, then steady-state TMP can be obtained as:

$$\Delta\psi_E = -1.5r|\mathbf{E}|\cos\theta \quad 2.6$$

Other than TMP, cells also have resting potential, which is typically in the range of -40 to -60 mV due to the ionic concentration gradient present inside and outside of the cell membrane. Therefore, the total TMP can be written as:

$$\Delta\psi_{total} = \Delta\psi_E + \Delta\psi_{rest} \quad 2.7$$

As the resting potential is negative in nature, electroporation initially occurs at the pole closest to the anode where the pole is hyperpolarized.

2.3 Theory of electroporation

This section introduces the theory of electroporation. In the literature, two main theories are reported regarding electroporation-induced pores on the cell membrane: the electromechanical compression theory and the aqueous pore model.

2.3.1 Electromechanical compression theory

Zimmerman proposed the electromechanical compression theory as a model for the dielectric breakdown of the cell membrane (Jiang et al. 2016; Zimmermann et al. 1974). In this model, pores are the result of electromechanical collapse due to compression of the entire cell membrane (Crowley 1973; Weaver and Chizmadzhev 1996). Here, the cell membrane is treated as an elastic capacitor that consists of a dielectric. Upon the application of electric voltage U , electric pressure is developed on this dielectric, which is given as:

$$p_{el} = \frac{\epsilon_m U^2}{2h^2} \quad 2.8$$

where ϵ_m is the permittivity of the dielectric and h is the thickness of the membrane. With the assumption that membrane compression is independent of change in thickness dh , the elastic compression force per unit area (p_m) of the membrane is written as:

$$p_m = -Y_m \int_{h_0}^h \frac{dh}{h} \quad 2.9$$

where Y_m is Young's modulus of elasticity, and h_0 represents the initial thickness when $U = 0$. Under equilibrium, electric pressure is balanced with elastic force as:

$$Y_m \log \frac{h_0}{h} = -\frac{\epsilon_m U^2}{2h^2} \quad 2.10$$

Assume that the breakdown occurs at voltage $U > U_c$, where U_c is the breakdown voltage such that $U_c^2 = 0.368Y_m h_0^2 / \epsilon_m$. When $U > U_c$, there is no root for the

Equation 2.10. The model therefore predicts that breakdown occurs at $h/h_0 = 0.61$ for U_c of 5 V. This means that breakdown occurs if the voltage is sufficient to compress the membrane by 39% (Chen et al. 2006).

The model roughly predicts the membrane potential needed to break down the cell membrane; however, the experimental value shows a discrepancy because an electroporation effect can be obtained at much lower TMP. This model also assumes a constant Hooke's law even when the compression is too high. Another limitation of this model is that it uses a low Y_m value and neglects the change in capacitance with the change in thickness and assumes that the fate of the membrane depends only on the applied voltage, which contradicts with experimental results with varying pulse durations (White and Thompson 1973). This modeling approach is also unable to explain the stochastic nature of the membrane rupturing, giving rise to the aqueous pore model, which is based on energy.

2.3.2 Aqueous pore model

Aqueous pore model is widely accepted model for electroporation (Kinosita Jr and Tsong 1977; Movahed and Li 2011). In this theory, two types of pores are assumed to exist: hydrophobic pores and hydrophilic pores. Hydrophobic gaps are formed as a result of thermal fluctuation in the lipid bilayer of the cell membrane. As shown in Figure 2.4, hydrophilic pores have polar heads lined up in the wall region. Unlike hydrophobic pores, these pores allow the exchange of water molecules and ions across the membrane and also conduct electrical current.

Figure 2.4: Pore formation energy of a membrane for a single pore of radius r , plotted in arbitrary units. **(A)** Pore energy for a hydrophilic pore under no transmembrane voltage ($U = 0$) and under application of transmembrane voltage ($U \neq 0$). **(B)** Two branches of pore energies: ΔW_1 , hydrophilic pore; ΔW_2 , hydrophobic pore. **(C)** Schematic of hydrophilic (top) and hydrophobic pore (bottom). Reproduced with permission from (Weaver and Chizmadzhev 1996), copyright 1996, Elsevier

The creation and destruction of hydrophilic pores is based on bilayer energy. In this model, first a pore free membrane is considered, and then a circular cut is made on the membrane, and finally the difference in the energy between these two states is calculated. Suppose γ represents the energy per unit length along the circumference required to create the pore, and Γ is the energy per unit area of a pore-free membrane, then the difference in the energy between these two states under no applied voltage is given by (Efimov et al. 2008; Weaver and Chizmadzhev 1996):

$$\Delta W_p(r) = 2\gamma\pi r - \Gamma\pi r^2 \text{ at } U = 0 \quad 2.11$$

However, considering the electrical energy, the pore can be associated with having an energy with change of specific capacitance C_{LW} from lipid to water. Although lipid is replaced by water, the resistance of the pore would remain high compared to spreading resistance such that the voltage across the pore is close to U , and this resistance is given by $R_p = \rho_e h / \pi r^2$. Therefore, under the presence of an electric field, the free energy for creating a pore is written as:

$$\Delta W_p(r, U) = 2\gamma\pi r - \Gamma\pi r^2 - 0.5C_{lw}U^2\pi r^2 \quad 2.12$$

Similarly, when water displaces lipid from the pore, the change in specific capacitance can be modified as:

$$C_{lw} = \left(\frac{\varepsilon_w}{\varepsilon_m} - 1 \right) C_0 \quad 2.13$$

where ε_w and ε_m are the permittivity of water and lipid, respectively, and C_0 is the capacitance of a pore-free membrane, i.e. $C_0 = \varepsilon_m/h$. As shown in Figure 2.4, the barrier height and corresponding pore radius decreases with increasing voltage, which is because of the higher permittivity of water compared to lipid. For a larger U , this height drops, increasing the probability of the membrane acquiring pores $r > r(U)_c$. This critical r_c is:

$$r_c = \frac{\gamma}{\Gamma + 0.5C_{lw}U^2} \quad \Delta W_{p,c} = \frac{\pi\gamma^2}{\Gamma + 0.5C_{lw}U^2} \quad 2.14$$

The values of the parameters used in this model can be found in the work from Efimov et al. (2008). This model is widely accepted compared to the electromechanical compression theory. However, the pore resealing process is not fully understood. Deng et al. (2012) modified this theory by adding one extra term involving stain energy, resulting in the following equation:

$$\Delta W_p(r, U) = 2\gamma\pi r - \Gamma\pi r^2 - 0.5C_{lw}U^2\pi r^2 + W_s(r) \quad 2.15$$

where W_s is the strain energy written with a six-order polynomial function of r for pore radius of 0.1–8 nm:

$$W_s(r) = \left(0.929 - 14.709r + 58.452r^2 - 44.998r^3 + 18.161r^4 - 2.724r^5 + 0.185r^6 \right) \times 10^{-21} (J) \quad 2.16$$

Their proposed model enabled resealing in reversible electroporation, and they claimed that the pore resealing took place via the release of stored strain energy in the membrane.

The aqueous pore model is widely accepted model for electroporation compared to the electromechanical compression model. In this thesis work, electroporation was achieved at transmembrane voltage as low as 0.2 V which is well predicted by aqueous pore model.

2.4 Bulk electroporation

As its name suggests, bulk electroporation (BEP) is carried out in bulk. In general, millions of cells are placed together with the cargo (gene or drugs) between two electrodes inside an insulated chamber. The electrodes are separated by a fixed distance (typically mm- or cm-scale). Figure 2.5 shows a conventional BEP system, where a cuvette consists of electrodes and a special chamber for loading the cells. Then, homogenous electric field strength is applied using high-voltage pulses (>1000 V, μ s or ms duration) to the sample. During this process, cells undergo electroporation and take in foreign materials. The electric voltage is chosen such that the threshold voltage is met to electroporeabilize the cell. Given the geometric constraints of the electroporation chamber, different electric fields are applied to different cells. However, electroporation efficiency is very low for such devices. Numerous cells are damaged due to the adverse environment created upon the application of high voltage. For example, bubble generation, change in pH and joule heating can damage the cells

permanently. Moreover, due to the high concentration of cells, electric field lines are also distorted and non-uniform, which can further reduce electroporation efficiency. Due to the high concentration of cells, some cells are shielded by the neighbouring cells, which prevents them from being electroporated. Based on these reasons, there is a heavy loss of drugs as well as genes, which is quite costly. Also, there is no control of the electroporation at the single-cell level. Moreover, in BEP systems it is difficult to deliver high-molecular weight cargo compared to small molecules, such as small interfering RNAs (siRNAs) (Chang et al. 2016b). As a result, many single-cell electroporation (SCEP) devices have been developed to address the limitations of BEP devices.

Figure 2.5 Conventional BEP system where millions of cells are electroporated inside a cuvette using high-voltage pulses

2.5 Single-cell electroporation

Recent advancements in microfabrication have made it possible to fabricate micro-electroporation chips that allow electroporation to occur at a much lower voltage than with BEP devices. Consequently, micro-electrical mechanical system (MEMS)-based

devices have been developed over the last few decades to carry out electroporation at cellular level. In this method, the basic principle is to generate a strong local electric field that can be focused on a single cell. Here, an inhomogeneous electric field is applied using microelectrodes. As a result, TMP can be achieved with much lower voltage. Moreover, microchannel flow control assists in positioning the cell precisely in the electroporation zones. This method also allows the minimum cargo to be loaded into the cells unlike in BEP, which requires high sample loads. Additionally, in SCEP, analysis can be done at cellular level, as the process can be monitored in a real-time environment.

2.5.1 Microfluidic electroporation devices

A plethora of devices has been developed for electroporating cells in microfluidic devices, including various electrode designs and the use of insulating structures. Here, these devices were classified into several categories, which are discussed below.

2.5.1.1 Devices with various electrode designs

Microfabrication technology has made it possible to design and fabricate different electrode shapes and sizes. As the electric field intensity depends on these factors, the design of these microelectrodes plays a critical role. Reducing the distance between electrodes can minimize the requirement of high electric voltage. Similarly, local high electric field strength can be generated using pointed electrodes. Hence, a suitable electrode design can improve electroporation efficiency.

a) Parallel electrode design

Parallel plate electrodes are the simplest electrode configuration used in microelectroporation devices. These electrodes, in general, mimic the electrode configuration of commercial electroporation systems. However, in these devices, electroporation can be achieved in flow conditions as well. Miniature versions of these

electrodes allow SCEP, where a homogenous electric field exists around the cell, which can result in higher transfection efficiency and higher viability. Some of these devices are presented in Figure 2.6.

Lin et al. (2001) fabricated a poly (methyl methacrylate) (PMMA) microchannel consisting of gold electrodes on the top and bottom of the channel. The device used much lower voltage compared to traditional cuvette-based electroporation systems and allowed continuous flow of the cell suspension. However, the fabrication process is complex, and the real-time monitoring of cell electroporation was not possible, as the electrodes blocked the view from the top and bottom of the channel. Miyano et al. (2008) constructed a parallel electrodes system modified to form micron-spiked electrodes to facilitate low-voltage electroporation. By using chemical etching, the precipitate in Fe-25Cr-6Ni alloy projected substantially, creating spikes with an extremely high aspect ratio. When an electric field was applied to those electrodes, these spikes produced multiple regions of high electric field strength, offering transfection of plasmid DNA into adherent cells.

Figure 2.6 Microfluidic electroporation devices with parallel electrodes, including (A) gold electrodes on the top and bottom of the microchannel. Reproduced with permission from (Lin et al. 2001), copyright 2001, Elsevier. (B) Micron-spiked parallel electrodes for enhancing electric field strength. Reproduced with permission from (Miyano et al. 2008), copyright 2008, RSC. (C) Parallel electrodes on a detachable paper-based device using wax bonding. Reproduced with permission from (Gong et al. 2010), copyright 2010, RSC. (D) Carbon nanotube (CNT)-based electrodes for enhancing the electric field. Reproduced with permission from (Shahini and Yeow 2011), copyright 2011, IOP Publishing. (E) CNT-coated substrate as bottom electrodes

and indium–tin oxide (ITO)-coated glass as top electrodes. Reproduced with permission from (Shahini and Yeow 2013), copyright 2013, RSC

In 2010, Gong et al. (2010) developed a simple, low-cost and detachable microfluidic chip with paper and glass slides with wax as a bonding material. Using wax bonding, the authors fabricated an electroporation chip consisting of parallel electrodes to electroporate HeLa cells. Their device achieved approximately 53% electroporation efficiency while maintaining 78% cell viability. Another modification to parallel plate electrodes was introduced by Shahini and Yeow (2011) in their work, where carbon nanotubes (CNTs) were integrated into a microfluidic electrolysis system. Using CNT-based parallel plate electrodes, the required voltage for cell lysis (for *E. coli*) was reduced by half while throughput was increased by 10 times compared to the device with non-CNTs. The use of CNTs increased the electric field strength based on the principle of the lightning rod effect. Another CNT-based electroporation device was developed by Shahini and Yeow (2013), where a CNT-coated substrate was used as the top electrodes, and indium–tin oxide (ITO) electrodes were used on the bottom. Using mammalian Chinese hamster ovary (CHO) cells, they demonstrated the release of intracellular medium using low-voltage electroporation. Their method achieved 72% electroporation efficiency with the voltage as low as 3 V.

b) Coplanar electrode design

This is also a common means of designing and fabricating microelectrodes for microfluidic electroporation devices. As the name suggests, the electrodes are considered 2D or coplanar. The electrode thickness is usually in the order of a few hundred nanometres scale. While the microchannel height is usually in the order of micron scale, these electrodes can be considered coplanar. Some examples of coplanar electrodes include rectangular electrodes, curved electrodes, interdigitated electrodes, and sawtooth electrodes and are presented in Figure 2.7.

Lin and Huang (2001) reported a microchip using thin-film electrodes and consisting of a defined cell culture cavity region. Using microfabrication, they fabricated 65- μm wide electrodes with a 200- μm gap. They reported the successful delivery of green fluorescent protein (GFP) genes to Huh-7 and 293T cells using 10 V voltage. Suehiro et al. (2003) described a method for extracting intracellular ions from bacteria by using both electroporation and dielectrophoretic impedance measurement (DEPIM) methods. Using an interdigitated microelectrode array, the authors used pDEP to capture bacteria, which was followed by irreversible electroporation for the ion extraction. The ions were detected as the increase in conductance of the sample. The authors could implement this method for detecting bacteria at concentrations as low as 10^2 colony-forming units (CFU)/mL. Suehiro et al. (2005) published an article for improving their method of electrophoresis assisted DEPIM for detecting bacteria. In that work, the authors implemented interdigitated electrodes for providing electrical excitation; however, in the current study, they improved the pulse waveshapes. Using different waveform shapes suppressed the electrolytic ions released from the metal electrodes, enabling secure detection of the intracellular ions released after the electroporation. In 2006, He et al. (2006) fabricated a micropulse radiofrequency electroporation (μPREP) chip with an array of 20×24 microchambers and comb-cross electrodes, where radiofrequency was used for electroporating cells. They fabricated 20-nm thin electrodes with 30- μm spacing between the electrodes. In their design, 12 different sets of electric conditions were applied, simultaneously reducing experimentation time by one order of magnitude. In that work, cabbage cells were successfully permeabilized, and fluorescent dye (propidium iodide, PI) was delivered. Using their method, the optimum conditions for electric field intensity can be determined in minimal testing time.

Figure 2.7: Coplanar electrode-based microfluidic electroporation devices including: (A) plain rectangular thin-film gold electrodes for delivering DNA into Huh-7 and 293T cells [reproduced with permission from (Lin and Huang 2001), copyright 2001, IOP Publishing]; (B) interdigitated gold electrodes for delivering GFP genes into Huh-7 and 293T cells and HUVEC (human umbilical vein endothelial cells) [reproduced with permission from (Lin et al. 2003), copyright 2003, Elsevier]; (C) rectangular coplanar electrodes for extracting intracellular protein from CHO cells [reproduced with permission from (Zhan et al. 2012b), copyright 2012, American Chemical Society]; (D) interdigitated electrode-based electroporation device developed for quick optimization of parameters [reproduced with permission from (He et al. 2006), copyright 2006, Elsevier]; (E) interdigitated electrodes coated with a thin layer of insulating materials to prevent the electrolytic effect on cell electroporation [Reproduced with permission from (Meir and Rubinsky 2014), copyright 2014, RSC]

Zhan et al. (2012b) developed an electroporation chip for extracting and analysing cellular proteins while keeping cells alive. Their device used two rectangular electrodes that were 500- μm wide and spaced 3 mm apart. Using 50-ms pulse of electric field intensity of 600 V/cm, a substantial fraction of the cell population was electroporated with the release of intracellular proteins from CHO cells while preventing cell viability. Lin et al. (2003) developed a coplanar interdigitated electrode-based microfluidic platform for electroporating adherent cells without detachment. Less than 8 V was used for the experiments, resulting in a survival rate of >31% and a GFP transfection rate of 13–36%. The paper concluded that a small DNA sample ($\sim 4 \mu\text{g}$) and low cell concentration (10^4 cells/mL) were sufficient for transfection, proving its significance over conventional BEP devices. The same group presented the use of interdigitated electrodes coupled for electroporation combined with electrophoresis for DNA transfer and concentration (Huang et al. 2007). Similarly, another microfluidic electroporation device that used interdigitated electrodes was reported by (Lin et al. 2004). The authors also used adherent cells for the experiments; however, electrostatic force was used at specific regions to attract plasmid DNA to the cells. The study simulated electric field distribution to understand the DNA attraction process. The authors concluded that electrostatic forces could assist in DNA plasmid enrichment at the anodes of the interdigitated electrodes, thereby increasing transfection.

The variation in electrode shape results in non-uniform distribution of the electric field, and therefore local high electric field strength can be generated. Figure 2.8 presents some of the devices that used different electrode configuration for enhancing electric field. Lee and Tai (1999) developed sawtooth electrode shapes to lyse different cells, including that of yeast, Chinese cabbage, radish, and *E. coli*. By reducing the electrode gap, they were able to reduce the voltage requirement to <10 V. Similarly, using

sawtooth electrodes, a non-uniform field was generated, with field strength of 1–10 kV/cm. The authors concluded that DC was able to lyse both protoplasts and microbes, while microbes were not lysed by AC at 1 MHz frequency (at 21 kV/cm). Lu et al. (2005) fabricated a microchip involving sawtooth electrodes for extracting intracellular components using electroporation. The tip-to-tip distance was 30 μm ; the groove-to-groove distance was 130 μm . Due to this variation, non-uniform distribution of the electric field was obtained, and up to 4.5 kV/cm field strength was obtained using <10 V. The study used an AC instead of DC field to prevent unwanted effects such as electrolysis. Moreover, the authors modelled the change in membrane potential for the cells as well as subcellular organelles with different frequencies. This was done to ensure that only the cells were lysed without affecting the intracellular components.

In 2008, a microchip that involved the use of sawtooth electrodes was developed by Sedgwick et al. (2008b). In that work, the authors combined DEP trapping with electroporation to transfect GFP to A431 human carcinoma cells. In the same device, on-chip cell lysis was also performed, and the protein release was observed using fluorescence microscopy. For trapping, the authors used 18 V_{pp} (volts peak to peak) at 1 MHz, while cell burst was observed at 20 V_{pp} at 500 kHz and 1 MHz. Nakayama et al. (2009) developed an integrated protein assay device, Single-Molecule MicroTAS (SMM), to lyse cells for extracting and purifying their proteins. For the cell lysis, the authors used sawtooth electrodes that were simulated with 50–250 V and a flow rate of 5 $\mu\text{L}/\text{min}$. In that work, protein was released from *E. coli* cells due to electroporation, followed by purification using a specific binding method. de la Rosa et al. (2008) developed a microchip using circular aluminium electrodes on glass to facilitate electro-manipulation of bacterial respiratory pathogens. In that work, the authors exploited pDEP in conjunction with pulsed voltage for manipulating and disrupting

cells. They used 10 V_{rms} at 1 MHz frequency to capture cells using pDEP, while 300 V DC pulses were used for electroporating cells. As a conclusion, they presented the electric field strength, the electrical conductivity of the sample, and the combined effect of pulse number and sample conductivity as the main factors that affected bacterial disruption.

Figure 2.8: Microfluidic electroporation devices with various electrode designs, including: (A) continuous converging and diverging electrodes (Wang et al. 2019); (B) curved rectangular electrodes [reproduced with permission from (Huang et al. 2011), copyright 2011, RSC]; (C) sawtooth electrodes [reproduced with permission from (Nakayama et al. 2009), copyright 2009, RSC]; (D) circular electrodes (de la Rosa et al.

2008); (E) sawtooth electrodes [reproduced with permission from (Lu et al. 2005), copyright 2005, RSC]

Similarly, Huang et al. (2011) proposed another curved electrode, where annular interdigitated electrodes were used. The microchip consisted of two juxtaposed chrome and gold layers on the glass substrate serving as electrodes. Using low electrical pulses (<100 V, <1 ms), their device achieved a cell transfection rate of $>90\%$. Furthermore, their device was compatible with the standard multi-well plates used in biological research, which presents new opportunities for high-throughput applications of siRNA technology. Recently, a novel method of determining the lethal threshold for bacterial electroporation was studied by Wang et al. (2019). They developed a device composed of platinum electrodes on glass that generated a linear gradient of electric field strength across the channel when voltage was applied. In their work, static experiments were conducted with *Staphylococcus epidermidis* bacteria. The LETs varied from 10 kV/cm to 35 kV/cm for different pulse conditions.

c) Three-dimensional electrodes

Most electroporation devices use either 2D electrodes or wire electrodes because of ease of fabrication. However, the electric field distribution is non-uniformly distributed around the cell, resulting in poor device performance. Recently, 3D electrodes have become increasingly popular in microdevices.

Lu et al. (2006) developed a 3D electrode array for cell lysis using electroporation. In that work, the authors investigated the effect of an electric field generated by 3D electrodes on cell electroporation and also compared the computational results with 2D devices. Using numerical simulation, they showed that using 3D electrodes enabled a higher effective volume ratio compared to 2D electrodes. Additionally, they demonstrated the formation of multi-pores as a result of the uniform electric field generated by the 3D electrode system, due to which the device efficiency increased to

30% compared to that of the 2D electrode device, which was 8%. Poudineh et al. (2014) developed sharp-tip 3D electrodes using electroplating. First, they fabricated thin-film gold electrodes using the standard lift-off technique. Then, electroplating was used to fabricate sharp-tip electrodes on the top of the thin-film electrodes. Using this method, the authors were able to lyse cells to extract the release of biomarkers without damaging the biomarkers. In 2017, Huang et al. (2017) developed a curved microchannel incorporating parallel electrodes on the top and bottom of the channel. Using the curved channel, the cell solution was enveloped with a protective buffer, eliminating electrolysis, joule heating and pH change near the electrodes. Additionally, at the channel outlet, both ions recombined and were neutralized due to Dean vortices. The method could be used for flow-through electroporation with high viability and high transfection rates.

d) Wire electrodes

Wire electrodes are usually incorporated by inserting two wires at the microchannel inlet and outlet. Platinum wires or silver/silver chloride (Ag/AgCl) wires are the most commonly used electrodes due to their chemical stability. Although microfabricated electrodes allow the generation of a high electric field at reduced voltage, these electrodes, once corroded, render the microchip unsuitable for reuse. However, wire electrodes eliminate this problem, as they are incorporated at the inlet and outlet ports. Insulator-based devices mostly use wire electrodes for inducing electrokinetic flow as well as for cell manipulation using DEP and electroporation-based lysis (Lee and Cho 2006). However, the use of such electrodes often requires a high voltage to ensure that a sufficient electric field is generated for electroporation (Wang and Lu 2006). In such cases, insulator-based devices with different geometrical configurations come into play, which will be discussed in the next section.

2.5.1.2 Insulator-based devices and constriction structures

Although the use of modified electrodes can enhance electric field strength, the detrimental effects of electroporation, such as pH change at the electrodes and electrolysis, can be observed (Lu et al. 2005). To minimize these effects, several insulator-based microfluidic devices have been developed, where electric field gradients are introduced using micro-insulators within the channel to locally enhance the electric field (iDEP devices).

Lee and Cho (2006) developed a lysis device that generates a high electric field using geometric constriction. The device operated on DC voltage, which generated electroosmotic flow (EOF). An orifice was used to focus the electric field such that a sufficient electric field was generated to lyse RBC. Similarly, outside the orifice region, the channel width was kept large such that a controlled EOF was generated. With an applied voltage of 50 V, the device could pump the sample with approximately 30 $\mu\text{m/s}$ velocity while maintaining 100% lysis efficiency. Similarly, Wang and Lu (2006) proposed a device where local field amplification was created using geometry variation. In the work, a reversible electroporation threshold for Chinese hamster cells was obtained as 400 kV/cm. Rapid cell lysis occurred when the field strength was increased to 600–1200 kV/cm. Besides, they also reported the equations for calculating the electric field strength in different sections of the channel as given below:

$$E_1 = \frac{V}{2L_1 + L_2(W_1/W_2)}, E_2 = \frac{V}{L_2 + 2L_1(W_2/W_1)}, \frac{E_2}{E_1} = \frac{W_1}{W_2} \quad 2.17$$

Another work to obtain the electric field threshold was proposed by Kim et al. (2007b), where a multi-channel electroporation microchip was developed. In that design, a single microchip could produce multiple electric field gradients by varying the lengths of the microchannel from 2 cm to 4 cm. Using five different channels of various

lengths, the authors obtained five different transfection rates in a single chip, which allowed the rapid optimization of experimental conditions. Geng et al. (2010) developed multi-orifice regions in a single channel, suggesting a means of eliminating the use of pulse generators. When cells pass through these multiple-orifice regions, they experience high electric field strength. The behavior is pulsed when they pass through multiple orifice in a series. In that work, the electric parameters of the electroporation were determined by the combination of electric voltage and the channel geometry and dimension. Further, the authors generalized the guidelines for scaling up these continuous flow devices.

In 2010, Church et al. (2010a) presented an integrated concentration and lysis of cells using a DC-biased AC field in a constriction-based device. The authors proposed using DC mainly to control the electrokinetic motion of the cells as well as to lyse the cells inside a constriction region where the electric field strength is high. Similarly, they proposed using AC mainly to control the cell concentration at the entrance of the constriction. They also optimized the DC and AC components to use for concentrating as well as lysing the cells. Bao et al. (2011) developed a flow-through electroporation method to obtain the time needed for individual cells in a group of RBCs to lyse under a specific electric field strength. In that work, they fabricated constrictions to amplify the electric field strength, whereas the flow was adjusted accordingly to obtain various residence times. The authors found that for 1.1 kV/cm intensity, RBC from normal, tropomodulin 1 (Tmod1)-null and β -adducin-null cells had different lysis times. Later, Kim et al. (2012) demonstrated multiple electroporation zones in a single channel. The chip consisted of a single microchannel with varying widths between the inlet and outlet. Due to these variations in width, multiple electric field regions were generated. The work enabled the optimization of the electric field threshold in a single channel. In

addition, the authors developed an equation for obtaining electric field values along the channel:

$$E_i = \frac{V_0 \rho}{RW_i h} \quad (i = 1, 2, \dots, n) \quad 2.18$$

where V_0 is the pulse voltage, ρ is the resistivity of the medium, R is the total resistance and W_i is the width of the i^{th} section in a microchannel.

Most of the abovementioned devices used DC field for electroporation in an insulator-based device. However, Zhan et al. (2012a) explored electrical lysis in the frequency range of 10 Hz to 10 kHz, because it was believed that under low frequency, TMP does not change with frequency. They demonstrated that electropermeabilization becomes weaker when the frequency increases. In addition, their experiments showed that transfection efficiency peaked in the range of 10 Hz to 1 kHz. Kim et al. (2014) developed a tapered channel to investigate the electroporation threshold. In their previous work, a stepwise channel was used, where the electric field strength was in discrete form. In the current work, they used a continuous converging channel to finetune the threshold field strength for H23 non-small cell lung cancer cells.

Recently, Bhattacharjee et al. (2016) explored electroporation at low DC voltage using microfluidic flipping of the voltage space topology. In that device, a serpentine channel was developed where electrodes were configured in such a way that each time cells pass through the constrictions, they experience the opposite field strength to that experienced in the previous channel. The authors reported increased electroporation performance as compared to previous flow-through devices. Similarly, Garcia et al. (2016) presented converging and diverging channels to determine the critical electric field for bacterial electroporation. Bilaterally converging channels were designed to amplify the electric field to the magnitude sufficient for inducing bacterial

electroporation. With that method, the authors were able to obtain a more precise threshold value for bacterial electroporation than other reported devices. In 2017, the same group developed a high-throughput electroporation device that comprised non-uniform constrictions in a microchannel that facilitated high field strength with low applied voltage (Garcia et al. 2017). The authors also compared their results with straight, converging, and diverging channels. They claimed that transformation efficiency could be increased by 4-fold and throughput by 100–1000 fold compared to the traditional method of electroporation.

2.5.1.3 Droplet-based electroporation devices

Hydrodynamic focusing is used for generating a narrow stream where the exchange of materials occurs. It allows a central flow to be sandwiched using sheath flows. Using a simple T-junction channel, Luo et al. (2006) constructed a picolitre-volume droplet system for electroporating yeast cells. Their design incorporates two planar gold (Au) electrodes, which provided size information and ion concentration change when low AC voltage was used. On the other hand, applying voltage of 18 V_{pp} at 1 kHz led to the irreversible electroporation of yeast cells inside a droplet. A similar design was later introduced by Zhan et al. (2009) to reversibly electroporate CHO cells using enhanced GFP. In their design, the main channel consisted of DNA and cells encapsulated in the droplets. These droplets flowed downstream to the pair of electrodes, where electric voltage was applied. In this region, the cells electroporated and took in GFP with voltage < 9 V for 20-μm spacing. Later, Xiao et al. (2010) constructed a droplet-based system using a T-junction configuration for electroporating HeLa cells. Their system also used a conventional T-shaped channel system, but with different dimensions to successfully generate 50-μm microdroplet encapsulated HeLa cells in oil phase. The

encapsulated droplets were collected at the outlet and mixed with fluorescent DNA dye in an electroporation cuvette chamber.

In 2012, Qu et al. (2012) established a co-flow-based droplet generation system where algal cells and foreign DNA were encapsulated in an oil phase. The device also incorporated the use of five pairs of electrodes downstream to enable electroporation of the cells inside a droplet. Additionally, a serpentine channel was used to facilitate better mixing of the contents inside a droplet. Their work enabled the transformation of cells at a low DNA to cell ratio at a reduced voltage. Further, they also investigated the effect of the cell wall on electroporation efficiency, where they used a no-wall mutant and a wild-type *Chlamydomonas reinhardtii*. They demonstrated that, compared to BEP, transformation efficiency for the no-wall mutant and the wild-type was >3 and >2 orders of magnitude greater, respectively. Recently, De Lange et al. (2016) developed a co-flow droplet-based microfluidic device for lysing *E. coli* cells using electroporation. However, electroporation was performed first, and the electroporated cells were mixed with lysozyme before generating droplets. The authors reported that electroporating the cells enhanced the lysis efficiency for cells that were incompatible with the lysozyme assay.

2.5.1.4 Nanoelectroporation system

Currently, nanoelectroporation systems are becoming popular among researchers. This is because working on a nanoscale allows users to create intense electric fields at much lower voltage. Thus, even a battery can power these devices to achieve electroporation. These systems allow the electric field to be focused on a specific area of the cell membrane where poration is needed, minimising the damage to the membrane. In the past few years, different nanoelectroporation systems have been developed, and include

2D nanochannel electroporation, nanospike electroporation, nanowire/nanoelectrode electroporation and 3D electroporation.

Boukany et al. (2011) were the first to develop a 2D nanochannel electroporation system for delivering precise amounts of transfecting agents into the cell without causing any destruction. The device was fabricated using combined standard lithography as well as DNA combing and imprinting. The device includes two microchannels connected by a nanochannel (90 nm in diameter). In the work, cells were placed in one microchannel while the transfecting agents were placed in the other microchannel. Upon the application of electric pulses, the cells experienced a strong electric field just at the edge of the nanochannel, causing nanoscale electroporation during electrophoretic movement of the cargo into the cytosol through the nanochannel, and the whole process could be controlled. In 2015, the same group published another paper on 2D nanochannel electroporation to deliver nucleic acids into target cells and also to detect their response at RNA level (Zhao et al. 2015). However, instead of using DNA combining and imprinting as in their previous work, the authors used silicon nanoridges and microridges to fabricate nanochannels and microchannels, respectively. Their platform could not only deliver cargo to the cells at single-cell level, but also provided quantitative analysis of multiple mRNAs/miRNAs (microRNAs) in a single cell without lysis. Furthermore, the group developed a modified version of their nanochannel-based electroporation device to greatly improve cell loading efficiency and viability (Gao et al. 2014). To do so, the authors first optimized a dip-combing device, producing DNA nanowires across the microridge array, and then shortened the length of the microchannel using femtosecond laser ablation.

Another nanoelectroporation system was developed by Xie et al. (2013), where the system was built on alumina nanostraws that extended from a track-etched membrane.

These nanostraws formed an array of hollow nanowires connected to an underlying microchannel. The work enabled electroporation at significantly low voltage due to cellular engulfment of the nanostraws providing intimate contact. Moreover, biomolecules were delivered by diffusion through the nanostraws, which was enhanced by electrophoresis upon pulsing. Similarly, Riaz et al. (2014) constructed an aluminium nanopike electroporation chip fabricated using nanoimprint lithography, electrochemical anodization and MEMS technology. Numerically, the authors observed enhanced local electric field strength at the tip due to the high aspect ratio of the nanopikes. With an applied voltage of 2.5 V, they demonstrated a highly efficient electroporation system along with high cell viability. The same group constructed an ultra-low voltage nanopike electroporation system that also incorporated a feedback system (Riaz et al. 2015). The authors presented several combinations for optimizing electric field parameters to obtain highly efficient electroporation of cancer cells with voltages as low as 1 V.

The other nanoelectroporation system includes the use of nanowires and nanoelectrodes. Jokilaakso et al. (2013) developed an ultra-localized SCEP that incorporated silicon nanowires. They reported a method for positioning the individual cells to the lysis region, which was silicon nanowire and silicon nanoribbon biological field effect transistors. Successful electroporation was demonstrated with an applied voltage of ≤ 0.6 V. Alternatively, several nanoelectrode-based devices have also been reported. Santra et al. (2014), for example, developed a pair of transparent ITO-based nanoelectrodes confined to a narrow region of the cell membrane near the edges of nanoelectrodes. This enabled the intense electric field to localize on a very small area of the cell membrane, leaving the remaining area of the membrane unaffected. In that device, a silicon dioxide (SiO_2) passivation layer was used on the top of electrodes to

further protect cells from the joule heating effect. Later in 2013, based on the same principle, Santra et al. (2013) constructed another device to deliver molecules into the cell while preventing damage. Moreover, they fine-tuned the electric field to increase efficiency as well as the cell viability in a controlled environment.

Lastly, 3D nanostructured surfaces for enabling electroporation are also well-reported. Kang et al. (2013) fabricated a nanofountain probe-based electroporation device to electroporate single cells. They developed a nanofountain probe cantilever tip that creates a localized strong electric field when in contact with the cell membrane. This method greatly minimized the effect of local joule heating while delivering molecules more precisely into the cells. The method achieved a high transfection rate (>95%) with >92% viability in HeLa cells. The same group developed another device using nanofountains to detect mRNA expression in live single cells using molecular beacon transfection. The authors used a DNA-based beacon during transfection for detecting glyceraldehyde-3-phosphate dehydrogenase, whereas an RNA-based beacon was transfected for detecting a sequence cloned in *GFP* mRNA (Giraldo-Vela et al. 2015). Other than nanofountains, 3D nanochannel electroporation was introduced by Chang et al. (2016a) that could transfect cells with extremely small molecules to large plasmids (>10 kbp). The device consists of an array of nanochannels that connect two microchannels. One microchannel is filled with cargo while the other contains the cells to be transfected. When the electric field is applied to the inlet ports of the two microchannels, strong field strength is developed at the entrance of the nanochannels that facilitate cell electroporation. The nanochannel array device could transfect >40,000 cells per 1 cm² at a time.

2.6 Electrokinetic effect in microfluidic devices

2.6.1 Electroosmosis

Electroosmosis (EOF) represents the motion of liquid under the influence of an external electric field across a channel. This phenomenon was first reported by F.F. Reuss in 1809, where he presented the motion of water through a plug of clay when an electric field was applied. In 1964, a mathematical model for EOF was formulated in a very fine capillary channel of the rectangular cross-section (Burgreen and Nakache 1964). In that model, the effect of electrokinetic radius for EOF was presented. Further, in 1965, Rice and Whitehead formulated equations for EOF inside a capillary (Rice and Whitehead 1965). Similarly, Keh and Liu performed an analytical study in 1995 to study steady-state EOF in a long uniform circular capillary (Keh and Liu 1995). They reported that solving the linearized Poisson–Boltzmann (PB) equation could obtain the electrical potential and the distribution of space charge density. Since then, many analytical, experimental or numerical studies have been performed for better understanding of EOF for channels of various sizes, shapes and configurations. Also, with the advancement of the lab-on-a-chip device, there have been numerous studies on EOF with implementation in the fields of biology, chemistry and medicine and engineering (Ferree and Blanch 2003; Gasparac et al. 2004; Kim et al. 2007a; Oddy et al. 2001).

The cause of EOF is the interaction of net surplus charged ions within a thin layer called the electric double layer (EDL) with an external electric field. In general, most surfaces acquire a surface charge when brought into contact with an aqueous medium. Some of the charging mechanisms include ion absorption, ionization and ion dissolution. Ions that have opposite charges to the surface are attracted to the surface.

These ions are called counterions. On the other hand, ions with similar charges to the surface (known as coions) are repelled. This process results in the formation of a thin layer near the surface, i.e. EDL. It is evident that there will be no electroneutrality within the EDL because the number of counterions exceeds that of coions. Consequently, a potential difference is developed within this layer. Figure 2.9 presents the schematics showing the distribution of charges and the formation of the EDL.

Figure 2.9 Formation of the EDL when the substrate encounters an aqueous solution (Outhwaite et al. 1980)

Near the wall, very thin layer is formed, called the Stern layer. The Stern layer is about one ion thick and is held very strongly with electrostatic force, rendering the ions immobile. A diffuse layer forms immediately after the Stern layer, in which the ion density follows a Boltzmann distribution. The electric potential varies from surface potential to the potential in the Stern plane and finally decays to zero far away in the diffuse layer. The potential in the diffuse layer drops exponentially in bulk solution. At the Stern layer, the potential is very close to the potential at a shear region, which is

well known as zeta potential. This potential indicates the shear plane for bringing fluid in motion upon applying an external electric field.

When an electric field is applied parallel to the surface, the excess ions within the diffuse layer result in a net body force to the fluid. This body force is proportional to the local net density of surface charge and tends to bring bulk fluid in motion. Moreover, the resulting electroosmotic velocity is directly proportional to the externally applied electric field strength, and to electroosmotic mobility, which is the function of both surfaces as well as solution properties.

Under the assumption of a thin EDL compared to channel geometry, a slip boundary condition is considered at the walls, which is expressed as:

$$\mathbf{u}_{eo} = \mu_{eo} \mathbf{E} \quad 2.19$$

where $\mathbf{E}$ is the externally applied electric field and μ_{eo} is the electroosmotic mobility of the bulk fluid and is given by the Smoluchowski equation (Tandon et al. 2008):

$$\mu_{eo} = -\frac{\epsilon_m \zeta_s}{\mu} \quad 2.20$$

where ϵ_m is the dielectric constant of the dispersion medium and ϵ_0 is the permittivity of free space. This relation is only valid for the thin double layer where $a\kappa \gg 1$, where a is the radius of the particle and κ^{-1} is the Debye length. For the thick double layer, $a\kappa < 1$; electrophoretic mobility is presented by Huckel's relation (Huckel 1924) as:

$$\mu_{eo} = -\frac{2\epsilon_m \zeta_s}{3\mu} \quad 2.21$$

2.6.2 Electrophoresis

When a charged surface such as that of colloidal particles is placed in a uniform electric field, it experiences electrophoretic force. This force drives the particles relative to the stationary liquid. The direction of the particle motion is determined by the direction of

the field as well as the type of charges the particle contains. Negatively charged particles move towards the cathode in the presence of anode and cathode terminals. In electrophoresis, the bulk motion of liquid is stationary. Electrophoretic velocity is determined as:

$$\mathbf{u}_{ep} = \mu_{ep} \mathbf{E} \quad 2.22$$

where $\mu_{ep} = \frac{\varepsilon_m \zeta_p}{\mu}$ is the electrophoretic mobility of the cell, with ζ_p being the zeta potential of the cell.

2.6.3 Dielectrophoresis

When a polarizable particle is introduced to a non-uniform electric field, it experiences DEP force. This time-averaged DEP force exhibited by spherical particles is given by (Pethig 2010):

$$F_{DEP} = 2\pi R^3 \varepsilon_m \operatorname{Re} \left(\frac{\varepsilon_p^* - \varepsilon_m^*}{\varepsilon_p^* + 2\varepsilon_m^*} \right) \nabla |E|^2 \quad 2.23$$

where $\nabla |E|^2 = \nabla (\mathbf{E} \cdot \mathbf{E}^*)$ is the gradient of the squared electric field, $f_{CM} = \frac{\varepsilon_p^* - \varepsilon_m^*}{\varepsilon_p^* + 2\varepsilon_m^*}$

is the Clausius-Mossotti (CM) factor. This DEP force is dependent on the electrical conductivity and permittivity of the suspending medium and particle, particle size, non-uniformity of the applied electric field, and the applied frequency. Based on the value of the CM factor, DEP can be classified into two types: nDEP (negative DEP) and pDEP. In nDEP, the particle moves from a high-electric field region to a low-electric field region, whereas in pDEP, the motion is just opposite. If the real part of the CM factor is negative, then the particle exhibits nDEP and pDEP for the positive value of the real part of the CM factor. Generally, at lower frequencies or DC fields, particles exhibit nDEP. However, if the frequency is sufficiently high, then the real part of the

CM factor becomes positive and particles exhibit pDEP. The velocity due to the dielectrophoretic movement of a spherical particle can be given as:

$$\mathbf{u}_{dep} = \frac{r^2 \epsilon_m \text{Re}[f_{cm}](\mathbf{E} \cdot \nabla) \mathbf{E}}{3\mu} \quad 2.24$$

where r is the cell radius, $\text{Re}[f_{cm}]$ is the real part of the CM factor that determines the nDEP positivity. The above expression indicates that the velocity of a particle under the influence of the DEP force is directly proportional to the volume of the particle and the non-uniformity of the electric field.

2.7 Application of electroporation on water treatment

Electroporation has proven its outstanding efficacy for inactivating microorganisms over conventional methods such as mechanical filtration, UV radiation and heat treatment. With the advancement in nanotechnology, high-aspect ratio structures such as nanotube and nanowire fabrication have become common. These high-aspect ratio structures made of conductive material can in fact significantly enhance electric field strength to magnitudes of several orders higher. One major disadvantage of an electroporation system is electrolysis, which ultimately leads to the formation of harmful electrolytic products. Therefore, the applied voltage should be reduced in such a way that the electrolysis threshold is not reached (~2 V). Moreover, reducing the voltage can significantly reduce the power consumption of the process. Hence, strong enhancement in the electric field is the current requirement in electroporation-based water treatment systems, and the role of these nanostructures comes into play.

Huo et al. (2017a) developed a pure CNT sponge using self-assembly. A self-assembly CNT (SACNT) sheet was first prepared by continuously drawing from an SACNT array on a wafer, which was then rolled into a multilayer film with a specific number of layers to form a sponge. The sponge structure had a pore size of 200–400 μm . The

authors used these two sponges as porous electrodes separated by a space. Outstanding bacterial inactivation performance was demonstrated with a voltage as low as 2 V. Earlier self-assembly-based fabrication methods were quite challenging. Therefore, Liu et al. (2013a) fabricated another nanosponge layer, where filters were developed using dip coating. The authors used highly porous polyurethane sponge and dipped it into CNT ink solution to coat it with CNTs. Moreover, they coated the sponge with silver nanowires (AgNWs) to enhance the nanoscale sharp tips. The device achieved up to 6 log removal efficiency with the applied voltage of 20 V and under high treatment speed of 15000 L/(h·m²). However, in this method, nanostructure coating was not uniform, so the field strength could not be controlled precisely. The same group demonstrated microbicidal electroporation using static electricity, which was powered by copper oxide nanowires (CuONWs) (Liu et al. 2014a). Using thermal oxidation, the authors grew nanowires on copper mesh at 500°C. This method produced uniformly distributed nanowires around the surface of the copper mesh. The application of 10 V showed >6 log reduction for *E. coli* samples. The device was powered by static electricity generated by rubbing plastic over aluminium foil. Huo et al. (2016a) used a similar fabrication technique to grow nanowires on the surface of a copper sponge. The authors examined the log removal efficiency under various voltage and flow conditions. At lower flow rates, high efficiency was obtained even at voltage of <2 V. In that work, the reduced voltage successfully eliminated the electrolysis problem. The same group developed a nanowire-assisted copper sponge to investigate the impact of several water quality parameters, such as ionic strength, pH and the presence of organic matter, in disinfection performance (Huo et al. 2018a). Due to high aspect ratios, the detachment of the nanowires became an issue and the nanowire-assisted electrodes could be sustained for only a few minutes (~10 min). To address these problems, the group

developed novel copper (I) phosphide nanowire (Cu₃PNW)-assisted copper mesh electrodes for water purification (Huo et al. 2018b). The nanowires were fabricated using the in-situ growth and phosphidation method. First, the authors used the anodization method to grow the copper hydroxide nanowires [Cu(OH)₂NWs], followed by phosphidation by placement in sodium hypophosphite solution. Thus, the formed structures showed robustness for a long time under flowing water and also showed enhanced performance during water treatment. Similarly, the group reported another method for improving the stability of the nanowires by introducing the concept of polydopamine (PDA) coating (Huo et al. 2019). Using solution-based self-polymerization, the authors produced a uniform and robust PDA layer with controllable thickness. Their method achieved >6 log removal of bacteria at 1 V, and the device could operate continuously for 16 h, which was around 100-fold longer than the working time of the uncoated device.

Nanotubes, nanorods and nanowires enhance the electric field based on the lightning rod effect. For example, when a metal rod is inserted into a uniformly distributed electric field region, the electric field near the tip of the rod can be estimated as:

$$\frac{|\mathbf{E}_{tip}|}{|\mathbf{E}_0|} = \alpha \frac{l_{rod}}{d_{rod}}, |\mathbf{E}_0| = \frac{U}{d} \quad 2.25$$

where $\mathbf{E}_{tip}$ and $\mathbf{E}_0$ are the enhanced and original electric field, respectively, l_{rod} and d_{rod} are the length and diameter, respectively, and α is a constant. By choosing the suitable nanostructure geometry, the required electric field strength can be obtained; however, too high an aspect ratio is undesirable, as the chances of breakage increases. Designing nanostructure-coated material for longer operation time is currently a challenge faced by nanoelectroporation systems for water treatment.

2.8 Summary

This chapter provides a broad literature review on electroporation, theories of electroporation and various techniques for achieving electroporation. In literature, various microfluidic techniques are reported to improve electroporation efficiency. Among the techniques discussed, the electrode-based devices and insulator-based devices stand out, as the electric fields therein are enhanced significantly compared to conventional BEP devices. Due to enhancement in the local electric field, the voltage requirement is minimized. However, for electrode-based devices, local pH change can have adverse effects on the cells, and the devices also experience the effects of corrosion. Similarly, current insulator-based devices include a large region of high electric field where the joule heating effect could be a disadvantage. Moreover, current methods are only suitable for applications with low throughput. Hence, the development of electroporation devices involving insulating structures like micropillars, microbeads, etc. are proposed, where multiple regions of local high field strength are established to achieve electroporation-based inactivation of microorganisms.

Chapter 3: Continuous flow microfluidic cell inactivation with the use of insulating micropillars for multiple electroporation zones

In this chapter, electroporation-based inactivation of yeast cells is reported using a microfluidic device that consists of an array of micropillars. The use of an array of micropillars generated multiple regions with enhanced local electric field strength. 2-folds increase of an electric field strength was obtained using pillar diameter to gap ratio (i.e., d/D ratio) of 2. Close to 63 % of cell inactivation was achieved which was nearly 2.5 times higher than a reference device without micropillars that was subjected to the same conditions. This chapter consists of four sections. Section 3.1 provides a background on cell electroporation along with several microfluidic devices developed for electroporation. This section also describes fundamental equations associated with the fluid flow and electroporation phenomena. Section 3.2 introduces our device geometry, cell preparation method as well as method for numerical simulation to achieve electric field distribution within the device. The role of micropillars in enhancing local field strength and its effect on cell inactivation is presented in Section 3.3. Finally, Section 3.4 concludes the results and discusses if the method can be used to electroporate cells for various applications.

3.1 Background

Electroporation is an effective method used in biological treatment with advantages that includes its high throughput, no usage of chemicals, and its effectiveness on a wide range of microorganisms (Adamo et al. 2013; Haberl et al. 2013; Lu et al. 2005). It is a non-viral technology commonly used for the extraction of subcellular organelles as well as for the transfection of genes and drugs (Gehl 2003b; Mahnič-Kalamiza et al.

2014; Weaver 2000; Yousuf et al. 2017). During the extraction process, nanopores are formed within the cell membrane upon the application of direct electric field and when a threshold transmembrane potential is met (Adamo et al. 2013; Gehl 2003b; Haberl et al. 2013; Lu et al. 2005). In earlier days, bulk electroporation (BEP) devices were used for electroporation where cells were placed inside a cuvette with two inserted electrodes (Weaver 2000). In those devices, high voltages were required to generate sufficiently strong electric field to electroporate the cells. Apart from the voltage requirement, these BEP devices cannot do single-cell handling and manipulations (Santra and Tseng 2013b).

Nowadays microfluidics has a wide range of applications because it can provide precise control of experimental conditions (Das et al. 2017; Lewpiriyawong et al. 2010, 2012; Zhao and Yang 2012). Microfluidic devices allow for isolation, trapping, merging and electroporation of a single cell. Furthermore, strong local electric field can be generated at much lower voltage. In the literature, many single cell electroporation devices have been reported for studying cell transfection (Lin et al. 2001; Lin et al. 2003) and cell inactivation (Fox et al. 2005; Knorr et al. 2001; Pol et al. 2000) as well as cellular content and cellular properties (Suehiro et al. 2005; Suehiro et al. 2003; Valero et al. 2005). Early microchips utilized a single pair of coplanar electrodes for electroporation of cells but there is a fast decay of the electric field strength away from the electrodes, resulting in poor device performance. To resolve this issue, other types of electrodes were fabricated, including interdigitated electrodes, rectangular, circular (de la Rosa et al. 2008), curved (Huang et al. 2011) and sawtooth (Nakayama et al. 2009; Sedgwick et al. 2008a).

Similarly, different channel geometries such as converging and diverging microchannels were developed to improve the electroporation efficiency as well as

throughput (Garcia et al. 2017; Garcia et al. 2016). The use of such geometries has shown advantages over the use of modified electrodes, such as minimizing the effects of pH condition and electrolysis on electroporation (Lu et al. 2005). Several other techniques incorporating nanowires and nanotubes to enable low voltage electroporation have also been reported as well (Huo et al. 2017b; Huo et al. 2016b; Liu et al. 2013a; Liu et al. 2014a). Recently, iDEP based microfluidic devices have shown many attractive advantages in single cell manipulations (e.g., isolation, separation and concentration) (Gallo-Villanueva et al. 2009; Moncada-Hernandez et al. 2011). In these devices, nonuniform electric fields are obtained by using insulating microstructures (Martinez-Duarte 2012). As a result, locally enhanced electric fields are established, thereby facilitating cell electroporation applications.

A continuous flow microfluidic device for enhancing the local electric field to achieve better continuous inactivation of the cells was reported. An array of insulating micropillars was introduced inside a microchannel to create multiple electroporation zones for generating sufficiently high local electric field strength. The aim was to inactivate microorganisms effectively even at a lower induced transmembrane potential (TMP) while maintaining high throughput. The inactivation performance of the device was assessed using a model solution of *Saccharomyces cerevisiae* (*S. cerevisiae*) under various electric field and flow conditions.

Figure 3.1 (A) Schematic of the microfluidic device with insulating micropillars used for electroporation-based cell inactivation. (B) Schematic illustration of the device working principle for inactivating yeast cells that are used in the experiment. (C) Cell electroporation device consists of PDMS micropillars for enhancing local electric field. Length, width, and depth of the fluidic channel are 2100 μm , 480 μm and 40 μm , respectively. Pillar diameter is 60 μm . Gap distance is 60 μm . (D) An enlarged view of the inactivation region.

3.1.1 Fluid Flow in a microchannel

Figure 3.1A shows the schematic of the proposed device with its working principle illustrated in Figure 3.1B. Considering a combined pressure driven and electroosmotic flow in the microfluidic device that comprises a microchannel with embedded micropillars. While modelling the fluid flow inside the microchannel, the flow field $\mathbf{u}$ of incompressible Newtonian fluid in such device is governed by the continuity equation $\nabla \cdot \mathbf{u} = 0$ and the steady-Stokes equation $-\nabla p + \mu \nabla^2 \mathbf{u} + \rho_e \mathbf{E} = 0$, where p is the pressure, μ is the dynamic viscosity of the liquid, ρ_e is the net charge density and $\mathbf{E}$ is

the strength of externally applied electric field. Within the electric double layer (EDL), the net surface charge is non-zero, thereby leading to the electroosmotic flow. With an assumption of the thin EDL, the electrical body force term $\rho_e E$ can be dropped by considering a slip boundary condition at the channel walls (Xuan et al. 2004), and such slip velocity is expressed as Equation 2.19 (Barz and Ehrhard 2005), where the electroosmotic mobility of the bulk fluid and is given by Equation 2.20. Since net surface charge in the bulk region outside the EDL is zero and under the assumption of thin EDL compared to channel geometry, the electrical potential distribution ϕ is governed by the Laplace equation $\nabla^2 \phi = 0$, and the electric field strength is obtained using $\mathbf{E} = -\nabla \phi$.

3.1.2 Motion of cells in a microchannel

Cells moving inside the device also experience both electrophoresis because of their charges under the applied electric field, and dielectrophoresis because of their polarization under non-uniform electric field generated inside the micropillar region. Therefore, the motion of cells can be predicted by considering the combined effect of electroosmotic flow, electrophoresis, and dielectrophoresis. The velocity of a cell can be determined by (Hyoung Kang et al. 2006)

$$\mathbf{u}_p = u - \mu_p \mathbf{E} - \frac{c \epsilon_m r^2}{3\mu} (\mathbf{E} \cdot \nabla \mathbf{E}) \quad 3.1$$

where $\mathbf{u}$ is the fluid velocity under combined pressure driven and electroosmotic flow, μ_p is the electrophoretic mobility of the cell, c is a correction factor, and r is the cell radius. The correction factor is dependent on particle size and the channel dimension which can be determined by fitting the experimental data with the simulation results for particle trajectory (Church et al. 2010b). Under our experimental conditions, cells

experience negative DEP effect as the conductivity of biological cells is much lower than that of the solution (Church et al. 2010c). Since our experiments involved only DC field (frequency = 0), the Clausius-Mossotti (f_{CM}) factor can be calculated as

$$f_{CM} = \frac{\sigma_p - \sigma_m}{\sigma_p + 2\sigma_m} \quad 3.2$$

Under DC electric field, cells act as perfect insulator, $\sigma_p = 0$ which results in $f_{CM} = -0.5$. This indicates that cells experience negative DEP inside the device.

3.1.3 Cell electroporation

Additionally, cells undergo the electroporation effect when the electric field strength is sufficiently high. The conductivity of cell membrane usually is of about several orders lower than that of cytoplasm and external medium (Pucihar et al. 2001). Therefore, when the cell is introduced to an external electric field, its membrane acts as a capacitor and charges are induced across the membrane, giving rise to a potential, called as the transmembrane potential (TMP). Under a DC electric field $\mathbf{E}$, the induced TMP for a spherical cell is given by Schwan's equation as (Tsong 1991)

$$\Delta\psi_E = 1.5r|\mathbf{E}|\cos\theta \quad 3.3$$

where θ is the angle between the line of electric field and the line joining the center of the cell to the point of interest. This equation is valid as long as the conductivity of cell membrane is of about several orders less than the conductivity of the buffer medium (Pavlin et al. 2005). In the literature, successful electroporations were reported for lower conductive solutions such as DI water (0.055 $\mu\text{S/m}$) (Liu et al. 2013b), city water (5 mS/m) (Liu et al. 2014b), artificial buffer (1 mS/m, 5 mS/m, 0.14 S/m and 1.6 S/m) (Pucihar et al. 2001) and other buffer (127 mS/m) (Pavlin et al. 2005). According to a literature study (Pucihar et al. (2001)), the percentage of surviving cells increases with

decreasing medium conductivity buffer. Similarly, for a lower medium conductivity, a higher field is required to achieve the same amount of permeabilization (Ivorra et al. 2010). Referring to Equation A.19, TMP depends on f_s , which is strongly correlated with medium conductivity as given by Equation A.20. With a decrease in medium conductivity, f_s also decreases, and thus a higher electric field strength is required to achieve the same amount of membrane permeabilization.

When TMP reaches some critical value i.e. 0.2-1V, nanopores are formed within the cell membrane (Gehl 2003b; Santra and Tseng 2013b). These nanopores can reseal under normal condition when the electric field is removed, and the process is called reversible electroporation. In contrast, if the applied field is much higher than the critical value, the pores do not reseal, and the process is called irreversible electroporation. In the latter case, the cell membrane gets permanently perforated and the cell becomes permeable to suspended medium with the release of intracellular materials.

In this work, the use of micropillars generated multiple electroporation with high local electric field that electroporated cells irreversibly for TMP less than 0.6 V.

3.2 Materials and methods

3.2.1 Device design and fabrication

The channel design in this study consists of an array of micropillars (4×7) embedded in the microchannel. These micropillars are arranged in a staggered configuration; this in comparison with a squared configuration allows cells to be kept inside the lysis region for a longer time. In iDEP device, pillar geometry (shape and size) plays an important role in enhancing local electric field. Higher enhancement in the electric field can be obtained if the diameter of the pillars is increased for a fixed width of the

microchannel (i.e. by reducing the gap between micropillars) (Kale et al. 2013; Martinez-Duarte 2012). In our device, pillar diameter ($D = 60\ \mu\text{m}$) and gap ($d = 60\ \mu\text{m}$) were equal, and hence $d/D = 1$. Further enhancement in the electric field can be obtained if the diameter of pillars is increased by keeping the same width of the microchannel (i.e. $d/D < 1$).

Soft lithography technique was used for fabrication of the microfluidic device. A glass slide was cleaned using piranha etch, rinsed with DI water, and then dried with N_2 gas. Negative photoresist, SU8 2025 (MicroChem Corp., USA), was spin coated on the slide to achieve a thickness of $40\ \mu\text{m}$. Then, the slide was baked at 65°C for 3 minutes and for 6 minutes at 95°C . The slide was then exposed to ultraviolet (UV) light through a photomask and post-baked at 95°C for 6 minutes. It was cooled to room temperature and developed with a SU8 developer (MicroChem Corp., USA). The slide was rinsed with isopropyl alcohol (IPA), dried with N_2 gas and finally hard baked at 150°C for 5 minutes. PDMS (SYLGARD 184, Dow Corning) was mixed with the curing agent in the ratio 10:1 (%w/w) and degassed before pouring into the mold. It was then cured at 70°C for 1 hr. in a convection oven. The cured PDMS was peeled off from the mold and holes were made using a puncher (Miltex, Biopsy Punch). Finally, it was treated with air plasma (Plasma Cleaner, Harrick Plasma) for 3 minutes and bonded to a clean glass slide. The device was kept at 70°C in an oven overnight. Pt wires were inserted at the inlet and outlet for the electric connection. The distance between the two electrodes was 2.1 mm. Figure 3.1C shows a schematic of our micropillar based electroporation device and Figure 3.1D shows an enlarged view of the inactivation region. Figure 3.2 shows the actual electroporation chip connected with electrodes and fluidic tubes.

Figure 3.2: Cell electroporation chip with electrical connections and fluidic access. Wire electrodes are used as the electrical connections to the power supply.

3.2.2 Cell preparation

S. Cerevisiae cells were used as a model organism in our experiment. 2 mg of baker's yeast was rehydrated in 2 mL Yeast Extract Peptone Dextrose (YPD) broth for 2 hr in a shaking incubator at 30 °C. The cells were then concentrated by centrifugation at 5,000g for 5 minutes. The supernatant was removed carefully using a pipette, and the pellet was re-suspended in YPD. Cells were washed thrice with PBS solution and finally suspended in a PBS solution (diluted 1000 times). The electrical conductivity was measured to be 1.6 mS/m by using a conductivity meter (Accumet, Fisher Scientific). Cell concentration was measured by using a hemocytometer, and it was diluted to achieve the final cell concentration of about 10^6 cells/mL.

3.2.3 Cell electroporation experiments

Before conducting experiments, the microchannel was coated with 1% (w/v) Pluronic acid for 30 minutes, followed by air drying for an additional 30 minutes to prevent possible adhesion of cells on the channel walls. The cell suspension was then injected into the microchannel using a syringe pump. Electrical connection was made by

inserting 500 μm diameter Pt wires at the inlet and outlet ports where DC electric voltage was applied. At first, the flowrate was fixed at 4.5 mL/hr while the DC voltage was increased from 0 to 900 V to identify the optimum voltage for electroporation of cells. Since the purpose was to create electroporation, the maximum voltage supply was limited to 900 V to minimize the effects of Joule heating that may occur at higher voltage. Once the optimum condition for the applied electric voltage was obtained, the flowrate was increased from 2.5 mL/hr to 8.5 mL/hr while the DC voltage was kept at 900 V. As a comparison, a straight channel without micropillars was used as the reference device. Cells collected from the channel outlets were immediately re-suspended in YPB broth to prevent lysis due to osmotic pressure.

The conventional assay of propidium iodide (PI) staining was used to determine the percentage of perforated cells. PI molecules can penetrate the cells with perforated membrane and bind with DNA, thereby emitting strong fluorescence signals, while live cells show no response due to their intact membranes. 50 mg/ml PI was added to the sample and incubated in the dark for 30 minutes. The fluorescence images of cells were captured, at an excitation wavelength of 590 nm and an emission wavelength of 630 nm, by using an inverted fluorescence microscope (Nikon Eclipse TE2000, Nikon Instruments) and a CCD Camera (Vision Research). Images were then processed using ImageJ software (NIH, USA). Inactivation performance of the device can be characterized by the computing the percentage of PI-stained perforated cells as

$$\text{Inactivation performance}(\%) = \frac{\text{Total number of dead cells}}{\text{Total number of cells}} \times 100\% \quad 3.4$$

3.2.4 Numerical simulation

Due to the complex geometry of the device, electric field strength inside the device can neither be obtained analytically nor experimentally. Numerical approach was employed

to analyse the electric field. AC/DC module within COMSOL Multiphysics software was used to obtain the steady-state electric potential and electric field distributions. A 2D model was used to calculate the electric field distribution, $\mathbf{E} = -\nabla\phi$, from the Laplace equation, $\nabla^2\phi = 0$, where ϕ denotes the electric potential. The values for the dielectric constant and conductivity of the medium were 78 and 1.6 mS/m, respectively. The following boundary conditions were used: a) Specified electric potentials at the inlet and the outlet of the channel, b) Electrical insulation at all the remaining boundaries including micropillars. Three different electric conditions were studied with voltages of 300 V, 600 V, and 900 V.

Similarly, the laminar flow module was used to model the fluid flow. Using the N-S equations discussed in Section 3.1.1, flow distribution was obtained in the presence of electric field. This module was coupled to the AC/DC module by providing an electroosmotic slip velocity as the wall condition at all the boundaries except for the inlet and outlet, and specifically, a zeta potential value of -100 mV was used (Wu et al. 2005). Simulations were performed at different flowrates from 2.5 mL/hr to 8.5 mL/hr at the electric voltage of 900 V. During the post-processing, Equation 3.1 was solved to obtain the cell velocity as well as the trajectory of the cell.

The temperature distribution was also examined across the microfluidic channel by coupling the COMSOL AC/DC module to the Heat Transfer module. The temperature distribution in the microchannel is governed by

$$\rho C_p \frac{DT}{Dt} = \nabla \cdot (k \nabla T) + \sigma |\mathbf{E}|^2 \quad 3.5$$

where ρ is the density, C_p is the specific heat capacity, and k are the thermal conductivity of the medium (Kunti et al. 2019). 2D transient simulations were performed to obtain the maximum temperature in the channel. During the simulation, microchannel walls were considered as thermally insulating walls whereas inlet and

outlet were initialized with ambient temperature. The physical parameters used in this simulation are obtained from the reference (Kunti et al. 2019).

3.3 Results and discussion

3.3.1 Numerical results

a. Electric field distribution

Simulations results showed that micropillars were able to generate a highly non-uniform electric field. Figure 3.3A and Figure 3.3B depict the contour lines and surface plot showing the distribution of electric field for an applied voltage of 900 V. Similar distribution patterns of the electric field were also obtained for the other two conditions, i.e. 300 V and 600 V, but with lower electric field strength. As shown in Figure 3.4A, the maximum electric field strength occurs near the edges of micropillar walls. Each cell flowing through the device passes through multiple zones of high electric field strength where they are electroporated.

Figure 3.3: (A) Contour lines and (B) surface plots representing the distribution of electric field under an applied DC voltage of 900 V. (C) Comparison of the maximum induced electric field strength and (D) comparison of the maximum induced transmembrane potential (TMP) for two microfluidic devices: one with micropillars and the other without micropillars under different applied voltages of 300 V, 600 V and 900 V.

Quantitative values for maximum electric field strength were obtained and used for computing the maximum induced TMP. Figure 3.3C shows that the maximum electric field strength of about 9 kV/cm, 6 kV/cm and 3 kV/cm are induced for the applied voltages of 900 V, 600 V, and 300 V, respectively. As a comparison, the electric field strength was also calculated for the reference device, in which the electric field strength was calculated as $|\mathbf{E}| = -\Delta\phi/\Delta d$, where 'd' is the distance between two electrodes. A uniform electric field strength of 4.28 kV/cm of electric field strength was obtained for the applied voltage of 900 V.

Figure 3.3D shows a comparison of the maximum induced TMP for the cells passing through two devices, i.e., with and without micropillars. For the device with micropillars, the maximum induced TMP is less than 0.6 V under all the applied field conditions. However, for the reference device (without micropillars), the maximum induced TMP is noticeably lower.

b. Cell trajectory

To obtain cell's trajectory, Equation 3.1 was solved using COMSOL and the results are presented in Figure 3.4 (under the conditions of flowrate 2.5 mL/hr and DC voltage 900 V). It is seen that the influence of DEP force in particle trajectory seems to be insignificant.. This is because of relatively high flowrate used. Simulation shows the hydrodynamic force was found to be more dominant compared to electrokinetic forces.

Figure 3.4: Numerical simulation results for cell's trajectory and cell velocity for an applied DC voltage of 900 V and a flowrate of 2.5 mL/hr.

c. Joule heating effect

Under low-conductive buffer solution conditions, negligible Joule heating induced temperature rise in iDEP devices was reported by Gallo-Villanueva et al. (Gallo-Villanueva et al. 2019a). However, increasing the conductivity of the buffer solution

could lead to stronger Joule heating effect. The time taken for temperature development can be estimated using

$$\tau_T = \frac{D_h^2}{k/\rho C_p} \quad 3.6$$

Where D_h is the hydraulic diameter (i.e. 60 μm), k is the thermal conductivity of the medium (i.e. 0.598 W/m K at 23 $^{\circ}\text{C}$), ρ is the density of water (i.e. 997.62 kg/m^3 at 23 $^{\circ}\text{C}$) and C_p is the specific heat capacity of water (i.e. 4180 J/kg $^{\circ}\text{C}$ at 23 $^{\circ}\text{C}$). Using Equation 3.6, it can be show that $\tau_T = 25 \text{ ms}$ ($\tau_T \sim O(10^{-3})$). This implies that energy equation can be solved in pseudo steady state.

By solving the energy equation (i.e. Equation 3.5) numerically using COMSOL heat transfer module, the steady state temperature was obtained within a very short amount of time ($< 250 \text{ ms}$). The maximum temperature rise in the device was found to be less than 5 $^{\circ}\text{C}$. The thermal Peclet number can be obtained as

$$Pe = \frac{UD_h}{k/\rho C_p} \quad 3.7$$

where U is the velocity inside the microchannel. In this study, for the flow rate of 2.5 mL/hr and applied voltage of 900 V, $Pe \sim 15$. This suggest that the advection effect was quite dominant compared to the diffusion effect. Also, since the temperature rise is low, thermal effect on the flow and particle trajectory could be minimum. Figure 3.5A shows the maximum temperature over time for a flowrate of 2.5 ml/hr and an applied voltage of 900 V and Figure 3.5B depicts the temperature distribution at $t = 10 \text{ sec}$.

Figure 3.5: Numerical simulation results of the distribution of temperature field (in °C) for an applied DC voltage of 900 V and a flowrate of 2.5 mL/hr: (A) the maximum temperature over period of 2 sec (B) Steady state temperature distribution at 10 sec.

3.3.2 Effect on cell inactivation

Figure 3.6 shows the experimental results comparing the inactivation performance of the two different devices (i) with micropillars and (ii) without micropillars (reference) under an applied electric voltage of 900 V and a flowrate of 4.5 mL/hr. 56% of cells inactivation for the micropillar device and 24% of cell inactivation for the no micropillary reference device were found. For the micropillar device, the maximum induced electric field strength of 9 kV/cm was obtained, corresponding to 0.58 V of the induced TMP (see Figure 3.3D). On the other hand, for the reference device, only 4.28 kV/cm of electric field strength was obtained, corresponding to 0.24 V induced TMP. In addition, the induced TMPs for both devices reside within the range of critical TMP. Hence, even though the reference device had relatively low inactivation efficiency, cell inactivation was still observed.

Figure 3.6: Experimental cell inactivation results showing a comparison between two microfluidic devices: one with micropillars and the other without micropillars, under the conditions of the flowrate of 4.5 mL/hr and the applied voltage of 900 V. All quantification data are presented as the mean $\pm$ SD of three independent experiments.

3.3.3 Effect of applied electric field

The effect of applied electric field was investigated under voltages of 0 V, 300 V, 600 V and 900 V while keeping a fixed flowrate of 4.5 mL/hr. The results showed that electroporation occurred only when the induced TMP exceeded a threshold value of 0.2 V. At 300 V, electroporation did not occur because the induced TMP was lower than critical TMP, i.e. 0.2 V (see Figure 3.4D). Above 300 V, the electroporation effect was observed, and the performance of the device was increased by increasing the applied electric voltage.

Figure 3.7 shows a comparison of the fluorescence images of PI-stained cells (represent membrane perforated cells) for two microfluidic devices: one with micropillars and the other without micropillars, at a flowrate of 4.5 mL/hr and applied voltage of 900 V. Compared to the image for the reference device (without micropillars), the image for

the micropillary device shows that much greater numbers of the stained cells are visible.

Figure 3.7: Comparison of the fluorescence images of PI-stained cells for two microfluidic devices: one with micropillars and the other without micropillars, at a flowrate of 4.5 mL/hr and an applied voltage of 900 V. The white arrows indicate the PI-stained cells.

The effect of applied electric field on cell inactivation in the micropillar device is depicted in Figure 3.8. With increasing electric field, there is a significant increase in inactivated cells. The percentage of inactivated cells reached about 58 % under the conditions of 4.5 mL/hr flowrate and 900 V applied voltage (corresponding to the induced electric field strength of 9 kV/cm). Also, it can be noted that the irreversible electroporation was achieved even at a lower value of the induced TMP which in < 1 V (refer to Figure 3.3D). The reasons are due to the presence of i) multiple electroporation zones and ii) non-uniform electric field in the micropillary device, and these two can lead to a higher probability of the cells passing through the high electric field strength region and of the increased exchange of intracellular components.

Figure 3.8: Percentage of inactivated cells in the micropillary device under various applied electric voltages while keeping a fixed flowrate of 4.5 mL/hr. All quantification data are presented as the mean $\pm$ SD of three independent experiments.

3.3.4 Effect of flowrate

A series of experiments was performed under various flowrates, including 2.5 mL/hr, 4.5 mL/hr, 6.5 mL/hr and 8.5 mL/hr, while keeping a fixed applied voltage of 900 V. Flowrates below 2.5 mL/hr will result in bubbles being generated, resulting in an interrupted flow. This bubble generation might be due to the electrolysis of ions present in the solution. Also, due to use of high DC voltage in the experiments, electrolysis is more likely to occur. Figure 3.9 presents the percentage of inactivated cells in the micropillary device for various flow conditions at 900 V. For the flowrate of 2.5 mL/hr, the highest percentage of the inactivated cells of 62.64% was obtained. With increasing the flowrate from 2.5 mL/hr to 8.5 mL/hr, the percentage of inactivated cells decreases from 62.64% to 30.67%. This can be correlated with the cell retention time in the device. As the flowrate increases, cell retention time in the region of high electric field decreases, thereby reducing the time of cell permeabilization. However, cell retention time can be increased by adding a large number of pillars for improving cell inactivation performance.

Figure 3.9: Percentage of inactivated cells in the micropillary device under various flowrates while keeping a fixed voltage of 900 V. All quantification data are presented as the mean $\pm$ SD of three independent experiments.

3.4 Summary

Electroporation-based cell inactivation has been demonstrated in a microfluidic device that used insulating micropillar structures for enhancing local electric field and creating multiple electroporation zones. The device showed better cell inactivation performance over a reference device that had no micropillars. Moreover, results showed that the cells were inactivated even at lower induced TMP values, and the performance of the micropillar device increased with increased applied voltage. In the experiments, cells were inactivated at TMP less than 0.6 V (Typical value of TMP for irreversible electroporation > 1 V). In addition, results showed that with increasing flowrate, cells were more likely to survive under the same electric field conditions. Nonetheless, the micropillary device can continuously inactivate the cells with comparatively lower electrical voltages and can potentially be used in biomedical applications such as genetic engineering, synthetic biology, and biochemistry, as well as in environmental applications such as water treatment.

Chapter 4: Effects of micropillar shape and electric field type on bacterial inactivation using electroporation

Chapter 3 reported a microfluidic electroporation device using micropillars having pillar diameter to gap ratio (i.e., d/D ratio) of 2. Circular micropillars were considered, and experiments were performed using DC electric field. This chapter presents a microfluidic electroporation device with a d/D ratio of 0.15 where an electric field enhancement of 29 was obtained. Additionally, the chapter covers the experimental results obtained from with two different iDEP devices having different base geometries: (i) circular and (ii) rhombus. The effect of DC and AC voltage on the inactivation performance is also discussed in this chapter. Section 4.1 provides background knowledge on conventional methods of water treatment and their shortcomings, electroporation method, and few electroporation devices. The section also provides basic equations linked with fluid flow, electrokinetics and electroporation inside our device. In Section 4.2, design and fabrication of device is presented along with the methods for cell preparation, and numerical simulation. Both experimental and numerical results are presented in Section 4.3 which are summarized in Section 4.4.

4.1 Background

Today, inactivation of pathogens causing various water borne diseases is of critical importance. Common inactivation methods including chlorination, ultraviolet (UV) inactivation, ozonation, and membrane filtration have a number of disadvantages. Chlorination, the most commonly used low-cost inactivation method, produces carcinogenic by-products including N-nitroso dimethylamine and trihalomethanes (Lee et al. 2007; Mitch et al. 2003; Tang et al. 2014). The use of UV inactivation method is

limited due to its high costs of operation and maintenance, high energy consumption, and dependence on the turbidity of raw water (Guo et al. 2009; Guo et al. 2012; Krasner 2009). Similarly, the ozonation process requires extremely high electric field to generate ozone and is also costly (Gonçalves and Gagnon 2012; Tsolaki and Diamadopoulos 2010). Additionally, the membrane filtration method has several disadvantages such as the requirement of high pressure, membrane fouling after prolonged use, and higher maintenance cost (Gonçalves and Gagnon 2012). Recently, an inactivation device has been developed that can trap, inactivate and detect *E. coli* from water sample (Dasgupta et al. 2016). However, the method is unsuitable for the use in water treatment.

In our previous work, a micropillary device was presented for enhancing local electric fields to inactivate yeast cells using DC voltage, where pillar diameter to gap ratio (i.e., d/D ratio) was 2 (Pudasaini et al. 2019). This method shows a promising alternatives over conventional water disinfection methods as there is no formation of by-products, and cell reactivation is also prevented (Huo et al. 2016a; Liu et al. 2013a). However, the method needs optimization such that sufficiently strong electric field strength can be achieved with much lower applied voltage to induce electroporation effect. Recently, several microfluidic devices have been developed to study electroporation phenomena under controlled experimental conditions (Chang et al. 2016b; Santra and Tseng 2013a). Furthermore, to enhance the electric field strength, techniques such as the use of 2D coplanar electrodes (Lin et al. 2001), sawtooth electrodes (Lu et al. 2005; Nakayama et al. 2009; Sedgwick et al. 2008b) and interdigitated electrodes (Adamo et al. 2013) have been reported. Similarly, several micropillary devices are reported in which electric field gradients are generated by using an array of micropillars within the channel to locally enhance the electric field (Pudasaini et al. 2019).

This chapter reports micropillary electroporation devices with a d/D ratio of 0.15 where an electric field enhancement as high as 29 was obtained using an AC voltage. Additionally, the effect of micropillar shapes on bacterial inactivation performance was investigated using two base geometries of micropillars: (i) circular and (ii) rhombus. The effect of DC and AC voltage was also examined along with the other parameters including applied frequency and flowrate on two different types of bacteria

4.1.1 Fundamentals

In our microfluidic device as presented in Figure 4.1, the motion of fluid is governed by the incompressible steady-state Navier-Stokes equations; $\nabla \cdot \mathbf{u} = 0$ and $-\nabla p + \mu \nabla^2 \mathbf{u} + \rho_e \mathbf{E} = 0$, where p is the pressure, μ is the dynamic viscosity of the liquid, ρ_e is the net charge density, and E is the applied electric field strength. The term $\rho_e \mathbf{E}$ can be dropped by assuming the electric double layer (EDL) thickness to be negligible compared to the microfluidic channel size. In this case, a slip boundary condition is used in the walls, and is given by Equation 2.19. Equation 2.20 presents the electroosmotic mobility of the bulk fluid. Under the thin EDL assumption, the electrical potential distribution ϕ is governed by the Laplace equation $\nabla^2 \phi = 0$, and the electric field strength is obtained as $\mathbf{E} = -\nabla \phi$. In the presence of electric field, cells also experience electrophoresis due to their charges and dielectrophoresis due to the induced polarization under non-uniform electric field. Electrophoretic velocity is obtained using Equation 2.22. The DEP velocity is expressed using Equation 2.24. In the equation, $\text{Re}[f_{cm}]$ is the real part of the Clausius-Mossotti (CM) factor that determines positive (attraction) or negative (repulsion) DEP. Overall, the cell velocity can be estimated by

$$\mathbf{u}_{cell} = \mathbf{u} + \mathbf{u}_{ep} + \mathbf{u}_{dep} \quad 4.1$$

where $\mathbf{u}$ represents fluid velocity component under combined pressure-driven and electro-osmotic flow (Hyoung Kang et al. 2006; Lewpiriyawong et al. 2012). Equation 4.1 can be solved using a numerical method to determine the trajectory of the cell.

Most microfluidic electroporation devices rely on the use of high voltages to manipulate particles/cells. The use of high voltages induces an electric current in the channel, producing a significant joule heating effect (Gallo-Villanueva et al. 2019b).

This joule heating effect can be serious if the medium conductivity is high. The temperature distribution in the channel is governed by energy equation as shown in

Equation 3.5. Volumetric joule heating $Q = \sigma |\mathbf{E}|^2$ is dependent on the conductivity of the medium (σ) and applied electric field strength ($|\mathbf{E}|$) (Sridharan et al. 2011).

In addition, the cell experiences the electroporation effect if the applied external field strength is sufficiently high. Since the conductivities of the cytoplasm and external medium are some orders higher than that of the cell membrane, when an electric field is applied, charges are accumulated across the membrane, resulting in a voltage difference, which is referred to as the induced transmembrane potential (TMP). When the TMP value reaches 0.2-1 V, the cell membrane is electroporated, leading to the formation of nanopores (Gehl 2003a; Santra and Tseng 2013a). For a DC field, this potential can be written as $\Delta\phi = 1.5r|\mathbf{E}|\cos\theta$, where r is the radius of the cell, $|\mathbf{E}|$ is the local electric field, and θ is the polar angle between the direction of the field and the line joining the centre of the cell to the point of interest (Tsong 1989). Usually, pores are formed at the location where $\cos\theta = 1$. This pore formation can be reversible or irreversible, depending on the intensity of electric field applied. In the literature, both electromechanical compression theory (Zimmermann et al. 1974) and pore energy model theory (Movahed and Li 2011) for electroporation are reported. Currently, pore energy model theory are widely used in modeling electroporation.

When a cell is placed in the AC field, the induced TMP is given by

$$\Delta\phi = 1.5r|\mathbf{E}|\cos\theta / (1 + (\omega\tau)^2)^{1/2} \quad 4.2$$

where τ is the time constant and ω is the angular frequency (Tsong 1989). Time constant τ is given by $\tau = rC_m(\rho_{\text{int}} + \rho_{\text{ext}}/2)$, where C_m is the membrane capacitance and ρ_{int} and ρ_{ext} are the resistivities of cytoplasm and external medium, respectively. To achieve the TMP threshold with the AC field, the voltage should last long enough to charge the membrane capacitance. Generally, low frequency is desirable such that $\omega\tau \ll 1$ (Mernier et al. 2010). For *E. coli* cells, τ is obtained as 1 μs . The parameters for *E. coli* cells were obtained from the literature (Hawkins et al. 2011; Park et al. 2011; Pucihar et al. 2001). Thus, the lysis frequency is $f \ll (1/2\pi\tau) = 158 \text{ kHz}$. In most of our experiments, frequencies of less than 10 kHz were used.

4.2 Materials and methods

4.2.1 Device design and fabrication of the device

Figure 4.1: **(A)** Schematic Schematic of our microfluidic electroporation device used for electroporation-based bacterial inactivation **(B)** Fabricated microfluidic device with embedded PDMS micropillars for enhancing local electric field. Length, width, and depth of the fluidic channel are 12 mm, 950 μm and 40 μm , respectively. Pillar diameter is 200 μm . Gap distance is 30 μm .

Figure 4.1A presents a schematic of our micropillary device used for electroporation-based bacterial disinfection. The device mainly consisted of a microfluidic channel, which is 12 mm in length, 950 μm in width and 40 μm in height along with embedded micropillars. Both circular and rhombus-shaped micropillars were examined. Both the diameter of the circular micropillar and the diagonal of rhombus were 200 μm , yielding a fixed d/D ratio of 0.15, with d being 30 μm . Both microfluidic devices were fabricated using a standard photolithography process followed by soft lithography. In short, SU8 photoresist was patterned on a silicon wafer with a thickness of 40 μm . After baking for 6 minutes at 95 $^{\circ}\text{C}$, the patterned wafer was exposed to a UV light through a photomask followed by a post-baking process. Finally, the mould was obtained by developing with SU8 developer. PDMS gel (10:1, %w/w) was poured into the mould and cured at 70 $^{\circ}\text{C}$ for 1 hour. Cured PDMS was peeled off the mould, holes were punched at the inlet and outlet, and the PDMS chip was bonded to a glass slide with aid of plasma treatment. Figure 4.1B depicts the circular micropillary device along with an enlarged view of the micropillars. The rhombus micropillary device is presented in Figure 4.2.

Figure 4.2: Fabricated iDEP device with embedded PDMS rhombus-shaped micropillars for enhancing local electric field. Length, width, and depth of the fluidic channel are 12 mm, 950 μm and 40 μm, respectively. Diagonal length is 200 μm. Gap distance is 30 μm.

4.2.2 Sample preparation

Two different bacteria, *E. coli* ATCC 25922 and *E. faecalis* ATCC 19433, were separately cultured overnight at 30 °C in a shaking incubator at 100 rpm. These cells were harvested using centrifugation at 5000g. Then the cells were washed twice using PBS solution and finally suspended in 0.9% NaCl solution as this solution prevents cell death due to osmosis. Most raw water contains Na⁺ and Cl⁻ ions abundantly. Tween20 (Sigmaaldrich, Singapore) was added to the sample containing cell suspension at 0.1 % (v/v) to prevent possible cell aggregation. The conductivity of the solution was adjusted to 5.5 mS/m by adding DI water.

SEM samples were prepared on a glass slide coated with poly-l-lysine (Sigmaaldrich, Singapore). First, cells were harvested using centrifugation and the sample was fixed with 2.5% glutaraldehyde (Sigmaaldrich, Singapore) at 4 °C for 30 minutes. A drop of the sample was placed on a glass slide for 10 minutes allowing cells to adhere on the surface. After adhesion, cells were washed twice with PBS followed by drying under various concentrations of ethanol, 35 %, 50 %, 70%, 95% and 100 %, each for 5 minutes. Finally, a drop of highly volatile HMDS (Sigmaaldrich, Singapore) was placed on the slide and left overnight. The entire drying process was carried out in a fume hood.

4.2.3 Experimentation

Prior to experiments, the microchannel was treated with 1% (w/v) Pluronic F127 (Invitrogen, USA) for 30 minutes to prevent cell adhesion to the PDMS channel walls. 1% (w/v) Pluronic acid solution was prepared by using 0.5 g of Pluronic F127 powders in 50 ml of DI water. After the surface treatment, the channel was flushed with DI water for 10 minutes. A syringe pump was used to inject the cell sample into the channel. Syringe needle were used as electrodes, and DC voltages were supplied using a DC power supply (Stanford Research). AC voltages were supplied by a function generator (Aligent, 33250A) and an amplifier (Optrobo, Singapore). Both voltages and currents were measured using a dual-channel digital multimeter. Cells were collected at the outlet, and cell viability was measured using a standard spread plate technique. Each sample underwent serial dilution and was plated on individual agar plates. These plates were kept in an incubator at 30 °C for 24 h. Concentrations of viable bacteria were obtained by counting the number of colonies, and log removal efficiencies were calculated as

$$\text{Log removal efficiency} = -\log_{10}(C / C_0) \quad 4.3$$

where C is the concentration of viable bacteria after treatment and C_0 is the initial bacterial concentration before treatment. Error bars were obtained based on triplicate experiments.

4.2.4 Electric field simulation

Due to the complexity of the geometry in our microfluidic devices, a numerical approach was deployed to obtain electric potential and electric field distributions using COMSOL Multiphysics software (Comsol, Burlington, USA). For an applied electric field of 250 V/cm, surface plots showing the distributions of electric field for both

circular and rhombus micropillary devices are presented in Figure 4.3A and 4.3B, respectively, for an applied electric field of 250 V/cm. An obvious enhancement in the local electric field can be observed in both devices. However, the maximum electric field strength in the rhombus micropillar device is notably higher than that in the circular micropillar device. At the sharp tips of rhombus micropillars, the Laplace equation has a singularity which causes a steep increase in the electric field, generating much higher field strength as compared to the circular micropillar. Electric field enhancements of approximately 13 and 29 were obtained for the circular and rhombus micropillar devices, respectively.

Figure 4.3: Comparison of electric field enhancement factor ($|\mathbf{E}|/|\mathbf{E}_0|$) for two microfluidic devices with: (A) rhombus-shaped micropillars and (B) circular micropillars. The darker region indicates higher magnitude of the electric field. Zoomed images show electric field contour lines (in kV/cm).

4.3 Results and discussion

4.3.1 Bacterial inactivation performance

Figure 4.4: Comparison of log removal efficiencies between two microfluidic devices with rhombus-shaped micropillars and circular micropillars under different applied AC electric fields; (A) for *E. coli* (gram-negative bacteria) and (B) for *E. faecalis* (gram-positive bacteria). All quantification data are presented as the mean $\pm$ SD of three independent experiments.

Figure 4.4A and 4.4B respectively show the bacterial inactivation performance of our devices for *E. coli* and *E. faecalis* under various applied electric fields (AC) while keeping the flowrate of 1 ml/hr. RMS voltages of 100, 200, 250, and 300 V were applied with frequency of 10 kHz across the inlet and outlet of the microchannel. These voltages correspond to the electric field strength of 83, 167, 208, and 250 V/cm, respectively. For the two devices, more than 3 log reduction of bacteria was obtained for both *E. coli* and *E. faecalis* under an applied field strength of 250 V/cm. Further, it can be observed that nearly 4.5 log removal was obtained for *E. coli* bacteria with the rhombus micropillary device. However, under the same conditions, only 3.5 log removal was gotten for the circular micropillary device. Similarly, for *E. faecalis*, log removal efficiencies were 4 and 3.4 for rhombus and circular micropillary devices, respectively. Remarkably, the rhombus micropillary device showed better performance

than the circular micropillary device for both bacteria. Comparative results from Figure 4.4A and 4.4B show that log removal efficiency for *E. faecalis* was lower than that of *E. coli*. This implies that to achieve the same magnitude of log reduction, *E. faecalis* requires a higher applied voltage than *E. coli*. This is probably due to the thicker peptidoglycan layer present in gram-positive bacteria like *E. faecalis*, whereas gram-negative bacteria like *E. coli* have a thin peptidoglycan layer (Huo et al. 2017a). Also, the size of *E. faecalis* is smaller than that of *E. coli*, and thus a stronger electric field is required to achieve the same extent of electroporation-based inactivation (Liu et al. 2014a; Tsong 1991).

SEM was used to visualize perforation in the *E. coli* cells after treatment to confirm that the damage is caused by electroporation. For SEM imaging, experiments were conducted for *E. coli* cells under the electric field strength of 250 V/cm. SEM imaging in Figure 4.5 shows poration in the cell membrane of *E. coli* cells. In the figure, nanopores can be observed. The average size of pores was found to be around 295 nm, measured using ImageJ software.

Figure 4.5: Demonstration of nano-pores formation on the cell membrane of *E. coli* cells using scanning electron microscopy (SEM) image. Applied electric field strength was 250 V/cm.

4.3.2 Characterization of device performance with AC and DC

Figure 4.6: (A) Comparison of log removal efficiencies of *E. coli* for various AC and DC electric fields. Flow rate is 1 mL/hr. (B) Effect of applied AC frequency on log removal efficiency of *E. coli* under the applied field strength of 250 V/cm and the flowrate of 1 mL/hr. All quantification data are presented as the mean $\pm$ SD of three independent experiments. (C) Transmembrane potential caused by the maximum field strength of 3 kV/cm under various frequencies.

Experiments were also performed to examine the effect of electric field type (i.e., AC and DC) on the device performance. Experiments were conducted with *E. coli* bacteria under various AC (frequency = 10 kHz) and DC voltages while keeping a fixed flowrate of 1 ml/hr. Only the circular micropillary device was used for this study. Results are depicted in Figure 4.6A, where it is clearly evident that the DC field shows higher log reduction than the AC field. Nearly 4.5 log reduction can be seen at the DC field of 167 V/cm, whereas only 3.5 log reduction is seen for the AC field of 250 V/cm. During our DC experiments, a significant amount of bubble formation was observed at the electrodes. Due to the presence of chlorine ions in our sample, there was a significant amount of electrolysis during DC experiments.

During electrolysis, NaCl is dissociated, and exists as Na⁺ and Cl⁻ ions in aqueous solution. Similarly, water also undergo reduction and oxidation reactions. Therefore,

both reactions are possible at both cathode and anode which are given below (Senftle et al. 2010):

At Cathode: reduction reaction: at pH =7

At anode: Oxidation reaction: at pH =7

However, for a very dilute NaCl solution, conductivity of the medium is very low. In small concentration, the electrolysis of water becomes more predominant yielding hydrogen at cathode and oxygen at the anode.

The total cell potential is thus, $E = -1.23 \text{ V}$ and is the minimum voltage needed for electrolysis (Bard et al. 1980). In the microfluidic electrolytic cell, the concentration of reactant reduces, and the concentration of product rises along the channel. In the absence of bubble formation within the microchannel, the electrolysis cell is modelled as a single pass, plug flow reactor. For a single pass, plug flow electrolytic cell, the fractional conversion, X , is given by (Green et al. 2015)

$$X = 1 - \frac{C_{out}}{C_{in}} = 1 - \exp\left[-\frac{k_m w L}{Q_v}\right] \quad 4.4$$

where c_{in} and c_{out} (mol cm^{-3}) are the concentrations of the electroactive species at the entrance and exit of the channel ($x = 0$ and $x = L$), respectively; k_m (cm/s) is the space averaged mass transfer coefficient, Q_v (cm^3/s) is the volumetric flow rate; w (cm) and L (cm) are the width and length of the channel respectively. It is seen that the theory

predicts a high conversion for the slower flow rate. The cell current, I_{cell} (A), required to achieve the fractional conversion, X , is given by (Green et al. 2015)

$$I_{cell} = nFQ_v C_{in} X \quad 4.5$$

where n is the number of electrons required for the chemical conversion at the working electrode; F (A s/mol) is the Faraday constant.

During experiments, an increase in the current was also noted over time, which is likely caused by an increase in conductivity due to electrolytic products. The increase in current was more significant at higher voltages. At levels above 167 V/cm of a DC field, bubble formation caused device failure due to clogging; therefore, experiments were not conducted for higher DC field strengths. Additionally, the corrosion of electrodes was also observed. However, in our AC experiments with 5 kHz frequency, no bubbles were observed even at 250 V/cm of an AC field, suggesting minimal effects due to electrolysis. For DC, only aqueous medium conducts electricity. As a result, most of the current are passed through medium and therefore more electrolytic products are possibly generated. However, for the AC case, both PDMS block and aqueous medium conducts electricity and therefore low current is conducted by aqueous medium suggesting lower electrolytic effect.

Figure 4.6B shows the effects of applied AC frequency on log removal efficiency for *E. coli* bacteria. The circular micropillary device was used for these experiments. At a fixed flowrate of 1 ml/hr and a fixed AC field strength of 250 V/cm, experiments were carried out for the various frequencies with linear steps of 2.5 kHz to 100 kHz (the maximum frequency that can be supplied by the equipment used). It was observed that log removal efficiency of bacteria is negatively correlated with the frequency. Upon increasing the frequency from 2.5 kHz to 10 kHz, log removal efficiency was slightly decreased from 4.05 to 3.46. Beyond 10 kHz, the log removal efficiency dropped

significantly. This is because the transmembrane potential does not change significantly for some frequency range (refer to Figure 4.6C). Beyond 10 kHz, there is a noticeable drop in TMP, and the ability of the cell membrane to be permeabilized is reduced upon increasing frequency. At 100 kHz, the efficiency was reduced to 1.64. With increasing the frequency, the pulse length is decreased, which may result in lower exposure time in the region of the strong electric field. Furthermore, the efficiency might be altered by a synergistic effect of the frequency and the flowrate. Technically, a very low frequency AC frequency should converse to the data point under the DC field. For DC field or low frequency AC field, the transmembrane potential does not change significantly with the change in frequency. Therefore, the exposure time of the cell is always higher than the membrane charging time. Thus, the effect on electroporation should not change significantly. However, the motion of the fluid, as well as particle could be different for low frequency AC field compared to the DC field (due to electrokinetic effect) which might affect electroporation.

4.3.3 Effect of flowrate

Figure 4.7: Effect of flowrate on log removal efficiency of *E. coli* under the applied field strength of 250 V/cm using the rhombus micropillary device. All quantification data are presented as the mean $\pm$ SD of three independent experiments.

To examine the effect of flowrate on the inactivation performance of the device, experiments were conducted under different flowrates varying from 0.5 mL/hr to 2 mL/hr in the increment of 0.5 mL/hr. The rhombus micropillary device was used under the applied electric field of 250 V/cm (frequency = 10 kHz). At the flowrate of 0.5 mL/hr, bubbles were observed resulting in the blockage of the channel. A decrease in the log removal efficiency was observed upon increasing the flow. In Figure 4.7, the highest log removal efficiency of 4.52 was observed for the lowest flowrate of 0.5 mL/hr, whereas the efficiency reduced to 4.11 for the highest flowrate 2.5 mL/hr. Increasing the flowrate decreases the holding time of the cell inside the region of the strong electric field. For the flowrate of 1 mL/hr, the residence time of the cell in the region of a strong electric field was found to be 1.25 ms (refer to Section 4.3.4). Additionally, because of the AC field used, cells should remain in place for sufficient time to allow for accumulation of voltage to charges for electroporation. In general, using the lower frequency of the AC signal and a lower flowrate can improve the inactivation performance of the device.

4.3.4 DEP response and particle trajectory

Clausius-Mossotti (f_{CM}) factor represents the polarizability of the particle/cell with respect to the surrounding medium, and is given by

$$f_{cm} = \frac{\epsilon_p^* - \epsilon_m^*}{\epsilon_p^* + 2\epsilon_m^*} \quad 4.6$$

where ϵ_p^* and ϵ_m^* denote the complex permittivity of the particle/cell or the medium respectively. Complex permittivity can be expressed as

$$\varepsilon^* = \varepsilon - j \frac{\sigma}{2\pi f} \quad 4.7$$

where $j = (-1)^{1/2}$, σ is the conductivity, and f is the frequency. For the DC field (frequency = 0), the Clausius-Mossotti (f_{CM}) factor can be calculated using Equation 3.2. Under a DC electric field, cell acts as a perfectly insulator, $\sigma_p = 0$, which results in $f_{CM} = -0.5$. This indicates cells experience negative DEP inside our device. However, for bacterial cells under AC field, the complex permittivity can be obtained using a two-shell model.. Thus, complex permittivity can be written as (Patel et al. 2012)

$$\varepsilon_{cw}^* = \varepsilon_1^* \frac{\left(\frac{r_1}{r_2}\right)^3 + 2\left(\frac{\varepsilon_{23}^* - \varepsilon_1^*}{\varepsilon_{23}^* + 2\varepsilon_1^*}\right)}{\left(\frac{r_1}{r_2}\right)^3 - \left(\frac{\varepsilon_{23}^* - \varepsilon_1^*}{\varepsilon_{23}^* + 2\varepsilon_1^*}\right)} \quad \varepsilon_{23}^* = \varepsilon_2^* \frac{\left(\frac{r_2}{r_3}\right)^3 + 2\left(\frac{\varepsilon_3^* - \varepsilon_2^*}{\varepsilon_3^* + 2\varepsilon_2^*}\right)}{\left(\frac{r_2}{r_3}\right)^3 - \left(\frac{\varepsilon_3^* - \varepsilon_2^*}{\varepsilon_3^* + 2\varepsilon_2^*}\right)} \quad 4.8$$

where ε_1^* , ε_2^* and ε_3^* are the complex permittivities of the cell wall, cell membrane and the nucleus. All the values for *E.coli* were taken from the reference (Hawkins et al. 2011; Park et al. 2011; Pucihar et al. 2001). A plot of CM factor with frequency is shown in Figure 4.8. For lower frequency, *E. coli* cells experience weak nDEP force under our operating condition.

Figure 4.8: The CM factor for live *E. coli* suspended in 5.5 mS/cm solution as the function of the frequency.

To obtain cell trajectory, Equation 4.1 was solved during post processing in COMSOL and the result is presented in Figure 4.9 (flowrate 2.5 mL/hr, 900 V). It is seen that influence of DEP force was negligible and no trapping effect was seen. Because of high flowrate used, hydrodynamic force was found to be more dominant in our device.

Figure 4.9: Numerical simulation results of the cell trajectory and the cell velocity for an applied AC field of 250 V/mm and flowrate of 1 mL/hr (frequency = 10 kHz) for (A) Circular micropillary device and (B) rhombus micropillary device. Equation 4.1 was solved during post processing to consider effects due to electroosmotic flow, electrophoresis and dielectrophoresis.

4.3.5 Joule heating effect

Joule heating effect was also analysed in our device using both physical scaling law analysis and the numerical simulation. Using Equation 3.6, the characteristic time scale was obtained as $\tau_T = 6.27 \text{ ms}$ ($\tau_T \sim O(10^{-4})$). Therefore, the energy equation can be solved in a pseudo steady state. Similarly, by using Equation 3.7, thermal Pe was obtained as 1.53. For scaling analysis, steady state was assumed, and negligible flow effect. Under these assumptions, temperature scales as $\Delta T \approx \frac{H_v R^2}{k}$ where

$H_v = \sigma E^2$, $R = d/2$, d is the gap between two micropillars where electric field intensity is maximum, $|E|$ is the electric field intensity and σ and k are the electrical and thermal conductivities of the medium respectively. Using physical scaling analysis, rise in temperature was observed to be $\Delta T = 13.41^\circ\text{C}$ for rhombus micropillary device and $\Delta T = 9.98^\circ\text{C}$ for the circular micropillary device.

Figure 4.10: Numerical simulation results showing steady state temperature distribution (in °C) for an applied AC field strength of 250 V/cm and flowrate of 1 mL/hr for both rhombus micropillary device (left) and circular micropillary device (right)

Similarly, by using heat transfer module in COMSOL, the steady state temperature distribution was obtained. Using Equation 3.5, temperature distribution was obtained. Flow simulation and electric field simulation were performed similar to the work from

Chapter 3 and are presented in detail in Section 3.2.4. Results are shown in Figure 4.10. Temperature rise of 4.2 and 3.6 °C was obtained at the outlets for rhombus and circular micropillary devices, respectively. During numerical simulation, flow simulation was also considered which might have resulted in lower temperature rise than scaling analysis. The initial temperature at the outlet was set to ambient temperature.

4.4 Summary

In this chapter, two different microfluidic electroporation devices are reported that can electroporate bacteria at a transmembrane potential lower than 1 V, enabling bacterial inactivation. Higher inactivation efficiency was obtained when rhombus-shaped micropillars were used. A numerical simulation was presented for obtaining the distributions of voltage and electric field. Numerical results identified the location of the maximum electric field between the two micropillars where electroporation occurs, and the results with rhombus pillars showed a higher maximum electric field obtained compared to circular pillars due to the sharp edges of the rhombus pillars. Similarly, DC field was found to be more effective than AC field for the frequency range studied. The electrolysis effect was significant while using a DC field. For the same AC voltage, the log removal efficiency lowered with increasing the AC frequency.

Chapter 5: An electroporation device with microbeads enhanced electric field for bacterial inactivation

Chapters 3 and 4 presented fundamental study on electroporation-based inactivation of microorganisms using microfluidic electroporation devices. In this chapter, a scaled-up electroporation device is presented where densely packed silica beads were used. Numerical simulation was conducted to obtain electric field distribution using unit cell model. In Section 5.1, several 2D and 3D microfluidic electroporation devices are presented. Design and fabrication of the device is discussed in detail in Section 5.2. In the same chapter, numerical simulation to obtain electric field distribution using unit cell model is also presented. Section 5.3 elaborates the results obtained from the experiments as well as from the numerical simulation. Finally, all the results are summarized in Section 5.4 and provides several areas of application where our method can be applied.

5.1 Microfluidic devices with 2D and 3D electrodes

Recently, various kinds of microfluidics devices have been reported to study electroporation under controlled experimental conditions (Chang et al. 2016b; Santra and Tseng 2013b). Early devices implemented the use of 2D electrodes, including rectangular (Lin and Huang 2001), interdigitated (Lin et al. 2003), saw tooth electrodes (Lu et al. 2005) and other shapes (de la Rosa et al. 2008; Huang et al. 2011; Wang et al. 2019) for generating high electric field strength. However, the electric field distribution is non-uniform because of the decay of field strength away from the electrode surface. To resolve this issue, the devices involving 3D electrodes were developed, and the electroporation efficiency was significantly improved (Huang et al. 2017; Lu et al.

2006). Although local high field strength can be developed using different electrode designs and configurations, the problems such as electrolysis and corrosion of electrodes were unavoidable. As a consequence, insulator-based microfluidic devices were developed where insulating structures (e.g., micropillars) with geometric variations are used (Church et al. 2010a; Garcia et al. 2017; Garcia et al. 2016; Kim et al. 2012; Kim et al. 2014; Wang and Lu 2006). The use of insulating structures not only enhances local electric field strength but also minimizes the adverse effects observed in conducting electrode-based devices. Insulator-based dielectrophoresis (iDEP) devices have been demonstrated for manipulating cells, particles as well as proteins (Gallo-Villanueva et al. 2011; Gallo-Villanueva et al. 2019b; Sano et al. 2013). In Chapter 3, a microfluidic electroporation device is presented involving to inactivate microorganisms under various electric field and flow conditions (Pudasaini et al. 2019). The device used insulating micropillars to concentrate and enhance electric field and thus minimising the need of high voltage for electroporation. Similarly, in the work presented in Chapter 4, electroporation was performed using two different microfluidic devices with various micropillar shapes. Experiments were performed on two different types of bacteria for various electric field conditions.

In this Chapter, an electroporation-based bacterial inactivation operating on an enhanced electric field induced by insulating microbeads is presented. Electric voltage was applied to a pair of mesh electrodes inside a chamber that consists of densely packed microbeads. Metal mesh electrodes are made of networks of periodic metal lines. The use of microbeads enhanced local electric field strength, at low applied voltages, that was sufficient to electroporate bacteria. The inactivation performance was tested for two types of bacteria: *Escherichia coli* (*E. coli*) and *Enterococcus faecalis* (*E. faecalis*) under various electric field conditions. Both of these bacteria are abundantly

found in raw water and are widely used as indicator organisms in the water treatment process (Kissinga et al. 2018; Worley-Morse et al. 2019). Figure 5.1A shows the schematic of our proposed device with its working principle illustrated in Figure 5.1B.

Figure 5.1: Design of a packed bead structure device for bacterial inactivation: (A) Schematic of the electroporation-based treatment device. 100 μm silica beads are packed inside a 2 mm hole in between two mesh electrodes (aperture = 50 μm). These mesh electrodes are sputtered with 25/400 nm Cr/Au; (B) Schematic illustration of the working principle of our device.

5.1.1 Fundamentals

In the device shown in Figure 5.1A, the motion of the fluid is governed by the continuity equation $\nabla \cdot \mathbf{u} = 0$ and the steady-state momentum equation $-\nabla p + \mu \nabla^2 \mathbf{u} + \rho_e \mathbf{E} = 0$, where p is the pressure, μ is the dynamic viscosity of the liquid. $\rho_e \mathbf{E}$ denotes the electrical body force with ρ_e being the net charge density and $\mathbf{E}$ the strength of the applied electric field. However, the electrical body force terms can be dropped off based on an assumption of a thin electric double layer (EDL); hence a slip boundary condition can be imposed (Xuan et al. 2004) and such slip velocity can be expressed by Equation 2.19 and the electroosmotic mobility of the fluid is given by Equation 2.20. Similarly, the electric potential distribution outside EDL region is governed by the Laplace equation $\nabla^2 \phi = 0$, and the electric field strength can be

obtained as $\mathbf{E} = -\nabla\phi$. With the presence of electric field, cells experience electrophoresis due to their electrostatic charges. In addition, the formation of non-uniform electric field generated inside the region of the packed bead results in cell dielectrophoresis effect. Therefore, considering the combined effect of electroosmotic flow, electrophoresis, and dielectrophoresis, the cell velocity can be estimated using Equation 3.1. Since the conductivity of the working fluid is much higher than that of cell membrane, cells experience a negative DEP effect (refer to Section 5.3.5). In most iDEP devices, joule heating effect can play a significant role as $Q = \sigma|\mathbf{E}|^2$, where Q is the volumetric Joule heating related to the electrical conductivity of the media (σ) and the electric field strength ($\mathbf{E}$).

Additionally, cells undergo the electroporation effect due to the charges induced across the cell membrane. These induced charges give rise to a voltage, termed as the transmembrane potential (TMP) which is given by Schwan's equation as (Tsong 1991)

$$\Delta\psi_E = fr|\mathbf{E}|\cos\theta \quad 5.1$$

where f is the shape factor and θ is the angle between the line of electric field and the line joining the center of the cell to the point of interest. Further, the shape factor is given by Equation 2.2 (Grahl and Märkl 1996). For the rod-shaped cell such as *E. coli*, $l \gg 2r$. Thus, $f = 1$. According to the theory of electroporation, when the transmembrane potential reaches the critical value of 0.2-1 V, , nanopores are formed within the cell membrane, thereby allowing exchanges of ions, drugs, molecules, genes, etc (Santra and Tseng 2013b). If the applied field strength is very high, the pores do not reseal, and the cell membrane is damaged permanently. Two major theories are reported in the literature; theory of electromechanical compression (Zimmermann et al. 1974) and the pore energy model (Movahed and Li 2011). Pore energy model theory are mostly used to model electroporation process. Schwan's equation remains valid as

long as the conductivity of cell membrane is several orders higher than the conductivity of suspending media (Pucihar et al. 2001). Successful electroporations have been demonstrated with various buffer conductivities ranging from DI water to saline solution (i.e. 1.6 S/m) without affecting the viability of the cell (Liu et al. 2013a; Liu et al. 2014a). However, the use of high conductive buffer could generate significant amount of heat which affect the electroporation process.

5.2 Materials and methods

5.2.1 Device design and fabrication

The device consisted of two acrylic blocks, one acrylic plate and a pair of mesh electrodes. A 15 mm hole was created at the center of 2 mm plate using laser cutting. Two stainless steel mesh wires, with a thickness of 0.5 mm and sufficiently large aperture ($\sim 50\ \mu\text{m}$), were cut into the desired shape (20 mm X 50 mm). These electrodes were sputtered with 25/400 nm Cr/Au to make chemically inert. Rectangular grooves were manufactured in the acrylic blocks to fit in the mesh electrodes. Figure 5.2 shows the assembled device with densely packed beads. Mesh electrodes were hooked up into the rectangular groove of both acrylic blocks. On the top of one block, the plate was attached, and the beads were packed. Silica beads ($\sim 100\ \mu\text{m}$) were mixed with deionized (DI) water to facilitate the packing process. A syringe was used to suck the water from the bottom to speed up the process. To ensure uniform packing process, beads were packed in a batch, and were levelled after packing each batch. Upon completion, another block was mounted from the top and was fixed with the screws.

Figure 5.2: Actual setup with an enlarged view showing the electroporation device with electrical connections and fluidic access.

5.2.2 Cell preparation

E. coli and *E. faecalis* were chosen as the modal organisms for the experiment. *E. coli* ATCC 25922 and *E. faecalis* ATCC 19433 were separately cultured overnight at 37° C in a shaking incubator at 100 rpm. The next morning, 10 µl of cell culture was added to 50 ml of fresh media and was grown to exponential phase ($OD_{600} \sim 0.5$). *E. coli* was cultured at Luria broth (LB) medium whereas *E. faecalis* was grown in a tryptic soy broth. Cell concentration was adjusted to $\sim 10^6$ cfu/mL by dilution. Cells were washed three times with sterile DI water and finally suspended in 50 ml of DI water with 0.1% Tween 20. 2 ml cell samples were collected before and after the treatment process. Concentrations of the sample were obtained using the standard spread plate counting method. After spread plating, samples are incubated overnight at 37° C. Results from treated samples and controlled samples were compared to obtain the inactivation performance of the device. Concentrations of live bacteria were measured by counting the number of colonies, and log removal efficiencies were obtained as

$$\text{Log removal efficiency} = -\log_{10}(C / C_0) \quad 5.2$$

where C is the concentration of live bacteria after electroporation treatment and C_0 is the initial concentration of bacteria before treatment

5.2.3 Experimental setup

Direct current (DC) voltages are supplied to the electrodes using a high voltage power supply HVS448 (LabSmith, Livermore, CA, USA) whereas alternating current (AC) voltages are applied using a signal from function generator and amplified by a high voltage amplifier. Experiments are conducted with different electric field strengths ranging from 0 kV/cm to 2 kV/cm. A peristaltic pump is used for generating the flow of cell suspension at a constant rate of 4 mL/min. For all voltage conditions, the constant flowrate was used. A magnetic stirrer was used at 100 rpm to make the suspension homogenous throughout the experiments. Before conducting any experiment, the device was flushed using ethanol and DI water. Further, to prevent the cells from adhesion, Pluronic F127 was used for coating channel walls and packed bead region.

5.2.4 Numerical simulation

COMSOL Multiphysics software is used to simulate the electric field distribution inside packed bead structures. Due to complexity in the geometry, a unit cell rhombohedral structure is considered for the numerical simulation. Figure 2 shows the workflow of our simulation. At first, the packed bead structure is developed (Figure 5.3A), and the unit cell is created using three microbeads (Figure 5.3B). Such unit cell is imported in COMSOL to obtain the simulation domain (Figure 5.3C). The domain is meshed using automatic features, and the following boundary conditions are applied (Figure 5.3D): a) Electric voltages on the anode and the cathode, b) Electrical insulation at the surfaces of microbeads and c) Symmetrical boundary condition for remaining boundaries. The values for dielectric constant and conductivity of the

medium are 78 and 5 $\mu\text{S}/\text{cm}$, respectively. An electric voltage of 40 V is applied to the anode whereas cathode is grounded. The distance between the electrodes is 100 μm , i.e. diameter of the beads. Above conditions for the unit cell are equivalent to our working device with the electrodes gap of 2 mm. Applied voltage was divided by number of layers where each layer is assumed to be the size of beads.

Figure 5.3: Numerical simulation workflow: (A) Computer-aided design (CAD) design of packed bed structure; (B) Development of a unit cell model using three beads; (C) Creation of the simulation domain in COMSOL; (D) meshing and boundary conditions

5.3 Results and discussion

5.3.1 Numerical simulation results

Simulation results show that the use of microbeads resulted in non-uniform distributions of the electric field with local enhancement in its field strength. Particularly, the results presented in Figure 5.4A and 5.4B show that the electric field strength reaches up to 18.5 kV/cm (near the corners of the cut plane). Additionally, the minimum electric field strength in the same cut plane was approximately 10 kV/cm which suggests at least 5 folds enhancement of electric field strength was achieved when beads were used with an applied field strength of 2 kV/cm. Numerical simulations were also performed to compare the electric field strength when no beads were used where the electric field distribution was uniform with its field strength of 2 kV/cm. Figure 5.4C compares the electric field strength for both devices (with and without beads), under various applied voltages. By using these electric field strengths, induced TMP can be obtained using Equation 5.4. Figure 5.4D shows the induced TMP for all operating conditions for both devices. For the device with beads, the induced TMP was greater than 0.2 V for all voltages higher than 100 V.

Figure 5.4: Distributions of electric field strength using COMSOL simulation for the device with beads under an applied voltage of 40 V (equivalent to the applied field strength of 2 kV/cm). Non-uniform distribution of electric field is achieved by using insulating beads. (A) Side and (B) top view are presented. Surface plots representing the distributions of electric field under various DC voltages. (C) Comparison of the induced electric field strength and (D) comparison of the induced transmembrane potential (TMP) for two devices: one with beads and the other without beads.

5.3.2 Inactivation performance of the device

The performance of our inactivation device was compared with the device with no beads. Experiments were conducted using *E. coli* for the applied field strength of 2 kV/cm with the flowrate of 4 mL/min. Figure 5.5 compares the log removal efficiency for both devices where a significant difference can be observed. More than 4.5 log removal (~ 4.75) was obtained when beads were used while the log removal efficiency is less than 1 (~ 0.57) when no beads were used. Under this electric field condition, the induced TMP for the device with beads was nearly 1 V (refer Figure 5.4D) which

favored electroporation. Whereas for the device without beads, the induced TMP was ~0.2 V (refer Figure 5.4D), under which minimum inactivation was achieved. This result suggests that the electric field can be enhanced significantly by using insulating beads to induce strong local electric field strength allowing higher electroporation rate. The energy consumption of the device was also obtained and was compared to the UV treatment method. For the flow rate of 4 mL/min and the applied potential of 400 V, the electrical current in our device was only 2.15 mA. This corresponds to the energy consumption of 12.90 kJ/L which is significantly lower than the energy consumption for UV disinfection methods (up to 933.6 kJ/L) for disinfecting bacteria (Keyser et al. 2008). Also, when beads were not used, energy consumption was 22.22 kJ/L for the applied voltage of 400 V and flowrate of 4 mL/min (current ~ 3 mA). The Energy consumption was obtained as

$$\text{Energy consumption} = \frac{U * I}{\text{Flux}} \quad 5.3$$

where U is the applied voltage and I is the current consumed. By considering the porosity of 0.36 for well packed spherical beads (Rhodes 2008), the volume of the treatment chamber was obtained as 0.636 cm³. Therefore, 6 sec hydraulic retention time (HRT) was obtained for the flowrate of 4 mL/min. Table 5.1 presents the energy consumption for the flowrate of 4 mL/min and the applied potential of 400 V.

Table 5.1 Energy consumption for our current device obtained for the flowrate of 4 mL/min and the applied potential of 400 V.

HRT (s)	Flux (mL/s)	Voltage (U)	Current (mA)	Energy consumption (kJ/L)
6.08	0.06	400	2.15	12.90

Usually, the treatment time for UV method is around 30-100 s which is much longer compared to our method (~ 6 sec) (Bai et al. 2012). The effectiveness of UV method

also depends on biomass concentration as well as turbidity of the liquid. Similarly, the requirement for periodic maintenance of lamp increases the cost of the treatment process. Our method also shows benefits over chemical methods of treatment as no chemicals were involved in this method, and the formation of by-products is also negligible (Huo et al. 2017a; Liu et al. 2013a; Liu et al. 2014a).

Figure 5.5: Comparison of bacterial inactivation performance with beads and no beads for applied field strength of 2 kV/cm at the flowrate of 4 mL/min. Quantification of colony-forming unit (CFU) was carried out using a standard spread plate counting method. $p = 0.0002$, using Student t-test.

5.3.3 Effect of applied electric field

Inactivation performance of the device was evaluated under various electric field conditions with the flowrate of 4 mL/min. Experiments were performed with various applied electric voltages of 0.5 kV/cm, 1 kV/cm, 1.5 kV/cm and 2 kV/cm. Control samples were collected for 0 V condition and used to compute log removal of bacterial concentration. After the treatment, all the samples were cultured for 18 hrs. in agar plates at 30 °C. As shown in Figure 5.6A, more than 4.5 log removal of *E. coli* was achieved for the applied field strength of 2 kV/cm. No viable cells were observed after 18 hrs. of culture. In the figure, it is observed that the number of viable cells decreases

for higher voltages due to the increase of the TMP for higher voltages. Similarly, log removal of *E. faecalis* was lower than *E. coli* under same electric voltage which is due to the presence of thicker layer of cell wall for gram-positive bacteria (Huo et al. 2017a).

Figure 5.6: Bacterial inactivation performance: Log removal in *E. coli* and *E. faecalis* concentration obtained for various applied electric field strength at the flowrate of 4 mL/min; (A) for DC electric field and (B) for AC electric field. All quantification data are presented as the mean $\pm$ SD of three independent experiments.

Similarly, experiments were also performed for various AC field strength ranging from 0.5 kV/cm to 2 kV/cm for both bacteria types. The log removal for both bacteria was lower compared to the results from DC (Figures 5.6A and 5.6B). For DC, voltage always remains at a same value while for AC, voltage changes with time, and the duration of the electric field gets short to charge the transmembrane potential to a level that induces electroporation. However, with the use of AC, total current was lower than that for DC. In addition, log removal efficiency is higher at low voltages for AC field compared to DC field, which might be due to DEP trapping effect (refer to Section 5.3.5).

5.3.4 Effect of AC frequency

To investigate the effect of AC frequency on bacterial inactivation, experiments were conducted with different frequencies at a fixed applied field strength of 1.5 kV/cm and flowrate of 4 mL/hr. Figure 5.7A represents the device performance for the frequency varying from 2.5 kHz to 60 kHz for *E. coli*. The log removal efficiency dropped from 2.5 to 1.5 when increasing the frequency from 2.5 kHz to 60 kHz. This is because the transmembrane potential does not change significantly in this range as represented in Figure 5.7B. In the presence of AC field, TMP changes with the applied frequency as given by Equation 4.2. Time constant τ is given by $\tau = rC_m(\rho_{int} + \rho_{ext}/2)$, where C_m is the membrane capacitance, and ρ_{int} and ρ_{ext} are the resistivities of cytoplasm and external medium, respectively. For higher frequencies ($>10^4$ Hz), the TMP reduces and the duration of electric field becomes short to charge the membrane, thereby causing the reduction of the electroporation (Huo et al. 2018b; Mernier et al. 2010). At higher frequencies, the log removal may be due to the cell DEP effect resulted from insulating beads induced non-uniform electric field.

Figure 5.7: (A) Inactivation performance of *E.coli* versus frequency with an applied AC field strength of 1.5 kV/cm (B) Transmembrane potential caused by electric field strength of 1.5 kV/cm at various frequencies. All quantification data are presented as the mean $\pm$ SD of three independent experiments.

5.3.5 DEP response

DEP is an electrokinetic transport mechanism with huge potential for the manipulation of bioparticles in microfluidic devices (Gallo-Villanueva et al. 2009). It is the movement of particles due to polarization effect in the presence of non-uniform electric field (Kua et al. 2008; Lewpiriyawong et al. 2011; Lewpiriyawong et al. 2012). In this study flowing cells experiences DEP effect due to presence of non-uniform electric field produced by microbeads. Thus, DEP response was also examined in this study. The time-averaged dielectrophoresis force experienced by the particles is given by (Pethig 2010)

$$F_{DEP} = 2\pi R^3 \epsilon_m \operatorname{Re} \left(\frac{\epsilon_p^* - \epsilon_m^*}{\epsilon_p^* + 2\epsilon_m^*} \right) \nabla |E|^2 \quad 5.4$$

where $\nabla |E|^2 = \nabla (E \bullet E^*)$ is the gradient of squared electric field, $f_{CM} = \frac{\epsilon_p^* - \epsilon_m^*}{\epsilon_p^* + 2\epsilon_m^*}$ is

the Clausius-Mossotti (CM) factor and $\epsilon^* = \epsilon - j(\sigma / \omega)$ is the complex permittivity.

Based on the sign of the CM-factor, DEP can be of two types: negative DEP (nDEP) and positive DEP (pDEP). For the DC field ($\omega = 0$) cases, The CM factor can be calculated using Equation 3.4. Under a DC electric field, cells act as perfect insulator, $\sigma_p = 0$, which results $f_{CM} = -0.5$. This suggests that bacterial cells experience nDEP inside our device under the DC electric field. While for an AC field, f_{CM} changes with frequency and is shown in Figure 5.8. The plot suggests that for the AC electric fields, bacteria experience pDEP for our operating conditions. Due to complexity in the device geometry, cell trajectory was not examined in this study.

Figure 5.8: The Clausius-Mossotti (CM) factor calculated for live *E. coli* suspended in DI water for various frequencies.

5.4 Summary

In this chapter, a new bacterial inactivation device using packed silica beads is presented that can generate high local electric field strength. The device serves as an efficient method of enhancing local electric field to achieve effective electroporation. The device showed better cell inactivation performance over a reference device without microbeads and it can continuously inactivate the microorganisms at much lower voltage compared to conventional electroporation systems and the method can potentially be used in different applications such as fresh, waste or ballast water treatment as well as disinfecting food products such as juice, milk, and wines.

Chapter 6: Insulator-based dielectrophoresis for simultaneous trapping and inactivation of microorganisms using contactless electrodes

In this chapter, a contactless DEP device is presented that can simultaneously trap the bacteria in the region of high electric field strength and perform electroporation. The use of sideways electrodes minimized the requirement of high voltage during operation. Furthermore, the use of contactless electrodes minimized problems that are commonly observed with electrode-based devices. Section 6.1 briefly discusses on DEP, their types and various application areas. This chapter also provides basic theory of cell trapping on iDEP devices. In Section 6.2, detailed design and fabrication of device is covered. Furthermore, electric model is also provided for obtaining the voltage drop across the medium and the results was verified using numerical simulation which is also presented in the same section. Section 6.3 discusses the experimental and simulation results for both pDEP trapping and electroporation. Section 6.4 explores the use of contactless DEP electroporation device for various applications.

6.1 Background on Dielectrophoresis and Electroporation

DEP is a widely used microfluidic technique for manipulating cells, particles and proteins for various applications (Pethig 2010). This phenomenon occurs due to the polarization of a dielectric particle in the presence of a non-uniform electric field (Regtmeier et al. 2011). This force depends on the relative properties of the particle and the suspending medium. In general, there are two types of DEP force: pDEP and nDEP (Pethig 2010). pDEP occurs when the polarizability of the particle is higher than that of

the suspending medium and the motion is towards the region of a high electric field. By contrast, nDEP is the motion of the particle towards the region of a low electric field and occurs if the suspending medium is more polarizable than that of the particle (Saucedo-Espinosa et al. 2016).

DEP only occurs in the presence of a non-uniform electric field, which are obtained using several means. The most common methods are via electrode-based and insulator-based devices. In the electrode-based DEP devices, electrodes of various shapes are incorporated to generate a non-uniform electric field. Interdigitated (Crews et al. 2007) and sawtooth (Hölzel et al. 2005) are some of such electrode shapes. However, in iDEP devices, insulating structures of various shapes are used to create a non-uniform electric field (Church et al. 2010a; Ferree and Blanch 2003; Gallo-Villanueva et al. 2019b; Saucedo-Espinosa et al. 2016). Usually in iDEP devices, the electric field is applied across the channel using wire electrodes. Due to the large distance, usually high voltage is required. Further, to manipulate particles, both DC and/or AC fields are used. DC fields are mainly used to control electrokinetic motion whereas AC fields are used for controlling DEP motion. Recently, iDEP devices have been developed for electroporating bacteria and yeast cells for water purification (Pudasaini et al. 2019).

Electroporation is the process of creating transient hydrophilic pores from initially formatted hydrophobic pores by permeabilizing the cell in the presence of an external electric field (Santra and Tseng 2013a). Electroporation can be used for permanent destruction of the cell membrane for extracting genes or other intracellular organelles from the cell or completely inactivate them (Santra and Tseng 2013b). For a DC field, this potential is written as $\Delta\phi = 1.5r|\mathbf{E}|\cos\theta$, where r is the radius of the cell, $|\mathbf{E}|$ is the local electric field, and θ is the polar angle between the direction of the field and the

line joining the centre of the cell to the point of interest (Tsong 1989). Pores are usually formed at the point where $\cos \theta = 1$. Similarly, the TMP for an AC field is given by Equation 4.2. Time constant τ is given by $\tau = rC_m(\rho_{\text{int}} + \rho_{\text{ext}} / 2)$, where ρ_{int} and ρ_{ext} are the resistivities of cytoplasm and external medium, respectively, and C_m is the membrane capacitance. Generally, low frequency is desirable such that $\omega\tau \ll 1$ (Mernier et al. 2010) to achieve the TMP threshold with the AC field such that the voltage remains sufficiently long to charge the membrane capacitance.

This chapter presents dielectrophoresis trapping of bacteria to achieve electroporation-based inactivation. The developed device here served a dual purpose by firstly squeezing field lines to define localized positive DEP traps and then establish region for bacterial electroporation. Using numerical analysis, strength of traps was presented at various electrical parameters. DEP method shows several advantages compared to patch clamp method. Although, patch clamp method is a powerful tool to investigate electroporation, the process is time consuming, laborious and allows serial analysis on small number of cells (Hübner et al.). In patch clamp method, accurate positioning of the micropipette on a cell is quite challenging (Wang et al. 2010b). Similarly, patch resistance may be influenced by the electrode filling solution, and is often much lower than is usually assumed (Fischmeister et al. 1986). In contrast, DEP method can position the cell accurately by fine tuning the relevant parameters allowing precise electroporation of cells (Luo et al. 2012; MacQueen et al. 2008; Wang et al. 2010a). In DEP, electroporation can be performed in a batch compared to patch clamp method. The patch clamp method is suitable for a single cell. For our application, high throughput inactivation of microorganisms is required where DEP based trapping for bacterial inactivation is advantageous. In this work, using insulator-based DEP device,

multiple electroporation locations were obtained enabling high throughput bacterial inactivation.

6.1.1 Theory

In iDEP devices, the electric field is typically applied across the channel through two electrodes: one at the inlet and the other at the outlet. Either a DC or both DC-biased AC field is usually applied. DC is mainly used to control the electrokinetic motion of the particle whereas AC is used for controlling DEP (Lewpiriyawong et al. 2012). In our contactless iDEP, flow is provided by using a syringe pump, and the AC electric field is applied through the liquid metal. Due to the presence of the AC electric field, particles experience DEP effect and the DEP velocity is expressed as:

$$\mathbf{u}_{dep} = -\mu_{dep} \nabla(\mathbf{E} \cdot \mathbf{E}) \quad 6.1$$

where μ_{dep} is the dielectrophoretic mobility and $\mathbf{E}$ is the electric field. Similarly, for combined pressure driven and electrokinetic flow, the flux of particle $\mathbf{j}$ is given by:

$$\mathbf{j} \cdot \mathbf{n} = 0 \text{ on the channel boundaries}$$

$$\mathbf{j} = \mathbf{u} + \mathbf{u}_{ek} + \mathbf{u}_{dep} \text{ in the microchannel} \quad 6.2$$

where $\mathbf{n}$ is the normal to the surface, $\mathbf{u}$ is the velocity due to pressure driven flow, $\mathbf{u}_{ek}$ and $\mathbf{u}_{dep}$ are electrokinetic and dielectrophoretic velocities, respectively. The condition for particle trapping is achieved for $\mathbf{j} \cdot \mathbf{E} = 0$ such that

$$\frac{\mu_{dep} \nabla I}{\mathbf{u} \cdot \mathbf{E} + \mu_{ek} I} \cdot \mathbf{E} > 1 \text{ in the microchannel} \quad 6.3$$

where $I = \mathbf{E} \cdot \mathbf{E}$ (Lapizco-Encinas et al. 2004). In the present study, pDEP was used for bacteria trapping in the high-electric field region to achieve electroporation-based inactivation. A numerical study was presented to illustrate the electric field distribution.

6.2 Material and methods

6.2.1 Device design and fabrication

Figure 6.1. (A) Schematic of our iDEP device used for electroporation-based bacterial inactivation. (B) Fabricated iDEP device consists of polydimethylsiloxane (PDMS) micropillars for enhancing local electric field. The length, width and depth of the fluidic channel is 12 mm, 950 μm and 40 μm, respectively. (C) The pillar diameter is 200 μm; the gap distance is 30 μm

Our actual fabricated microfluidic chip is shown in Figure 6.1A with its schematic in Figure 6.1B. The device consisted of a microfluidic channel of 12 mm in length, 950 μm in width and 40 μm in height. Two other side channels served as electrodes when filled with liquid metal, i.e. gallium–indium eutectic (Sigma-Aldrich, Singapore). These side channels were separated from the main channel by a 20-μm thin polydimethylsiloxane (PDMS) gap. The diameter of the micropillars was 200 μm, along with a 30-μm gap between them. Standard photolithography was used for developing an SU-8 mould (MicroChem, USA). In brief, a 40-μm thick SU-8 layer was

coated on a 4-inch silicon wafer and baked at 95°C. After cooling, the wafer was exposed to UV light through a photomask containing the pattern. After exposure, the wafer was baked again and then developed using SU-8 developer to obtain the final mould. After fabricating the mould, PDMS (SYLGARD 184, Dow Corning) was cast and cured at 70°C for 1 h. Then, the PDMS was peeled off and holes were punched followed by bonding to a glass slide by using plasma treatment bonding (Plasma Cleaner, Harrick Plasma).

6.2.2 Electrical modelling

To estimate the voltage across the micropillar region, the microfluidic device was modelled with an equivalent electrical circuit. Here, the channels containing liquid metal were modelled as a perfect conductor while the PDMS gap and medium were modelled as resistor–capacitor (RC) pairs in parallel, which were further connected in series (Pavesi et al. 2016). The capacitive impedance of the PDMS gap and medium were obtained using $Z_{cap} = 1/(2\pi fC)$, where f is the frequency and C is the capacitance, which is calculated as $C = \epsilon A/d$, where ϵ is the relative permittivity. Similarly, the resistance of the PDMS gap and medium was calculated as $R = l/(\sigma A)$, where σ is the conductivity and A is the cross-sectional area. A MATLAB code was written for an equivalent circuit and the voltage drop was calculated. The graph representing the change in voltage drop for various frequencies is shown in Figure 6.4D.

Table 6.1 Electrical parameters for PDMS and deionised (DI) water used for circuit modelling and numerical simulation (Shafiee et al. 2009)

Material	Electrical conductivity (S/m)	Relative permittivity
PDMS	0.83E-12	2.65
DI water	5E-04	80

Figure 6.2: (A) Equivalent electric circuit model for the microfluidic device. (B) Schematic of our iDEP device used for developing electric circuit modelling

6.2.3 Numerical simulation

Numerical simulation was used to obtain electric field distribution across the micropillar region with a complex geometry. The simulation was also used for obtaining the voltage drop across the medium, and the results were compared to that of

the electrical modelling. The AC/DC module of COMSOL Multiphysics was used to obtain the electric field strength by using the following equation:

$$\nabla \cdot (\sigma^* \phi) = 0 \quad 6.4$$

where ϕ is the externally applied voltage and $\sigma^* = \sigma + i\omega\epsilon$, where $\omega = 2\pi f$. A 2D model was developed in COMSOL. The edges of the liquid metal channel were assigned to the source voltage and ground. All materials were chosen from the COMSOL library, whereas the parameters were set using the reference (Shafiee et al. 2009).

6.2.4 Cell preparation

For the experiments, *E. coli* and yeast cells were used. *E. coli* was cultured at 30°C in a shaking incubator at 150 rpm, whereas baker's yeast was suspended in D-mannitol solution for 2 h. Cells were centrifuged and were washed with deionised (DI) water twice. Finally, the cells were suspended in NaCl solution containing 0.1% Tween 20 (v/v) to prevent the cells from clumping. The final conductivity of medium was 5×10^{-4} S/m.

6.2.5 Experimentation

The microchannels were coated with 1% (w/v) Pluronic F127 (Invitrogen, USA) for 30 min to prevent possible cell adhesion. The sample was injected into the microchannel using a syringe pump (kdScientific, Singapore). Electric voltages were applied to the reservoirs containing liquid metal using the AC signal of desired frequency (Tektronic, AFG-3022, USA) which was further amplified using an amplifier (OPT3, Singapore and Trek-623B, USA). The cells were stained using SYTO 9 (Thermo Fisher Scientific, Singapore) during the demonstration of trapping experiments. Figure 6.3 presents a calibration curve generated by measuring the fluorescence intensities for various

concentrations of *E. coli* stained with SYTO 9. An 8-bit camera with sensor value in the 0–255 range was used during the experiments. The calibration curve was used to quantify bacterial trapping by measuring the fluorescence intensity in the trapping region. For demonstrating the inactivation phenomena, SYTOX dye was used, which stains cells with compromised membranes.

Figure 6.3: Calibration curve indicating fluorescence intensity of SYTO 9 for various concentrations of *E.coli*

6.3 Results and discussion

6.3.1 Circuit modelling and numerical simulation

Figure 6.4A presents the electric field distribution at an applied voltage of 300 V_{rms} at 20 kHz; Figure 6.4B represents the electric field distribution at the same voltage but at 100 kHz. The maximum electric field inside the micropillar region was around 6 kV/cm for 20 kHz, whereas for 100 kHz, the maximum electric field was nearly 12 kV/cm. This shows that with increased frequency, the voltage drop across the medium was higher, as presented in Figure 6.4D. The results indicate that the frequency plays a

significant role in penetrating the electric current across the PDMS barrier in a contactless DEP device. The electric field gradient is shown in Figure 6.4C. Our device obtained an electric field gradient in the order of $\sim 10^{16}$, which is sufficiently high for bacterial trapping. Figure 6.4D shows the comparison of voltage drop across the medium between the numerical method and circuit modelling. The results indicate good agreement between the two methods.

Figure 6.4: Numerical results of the electric field and electric field gradient within the microfluidic device. **(A)** Surface plot representing electric field distribution for applied voltage of 300 V_{rms} at 20 kHz. **(B)** Surface plot representing electric field distribution for applied voltage of 300 V_{rms} at 100 kHz. **(C)** Surface plot representing electric field gradient for applied voltage of 300 V_{rms} at 100 kHz. **(D)** Line plots from electrical modelling and numerical simulation representing voltage drop across the medium under various frequencies

6.3.2 Bacterial trapping

Figure 6.6 shows the transient trapping of *E. coli* under the application of 150 V_{rms} at 20 kHz. Fluorescently labelled bacteria were used for the trapping experiments. The bacteria were trapped in the high-electric field region (refer to Figure 6.4a), indicating pDEP trapping. A plot of the CM factor under various frequencies is shown in Figure 6.5, which indicates for the medium used in the current experiments, *E. coli* experienced pDEP for a broad frequency range with a maximum value of 0.85 for the frequency of 100 kHz to 1 MHz.

Figure 6.5: Plot showing real values of CM factor under various frequencies for the medium conductivity of 5×10^{-4} S/m. 0.9% NaCl solution prepared and diluted using DI water to the desired conductivity.

Figure 6.6. Transient trapping of *E. coli* in the high-electric field region for different times: 0 s, 20 s, 40 s and 60 s for the applied voltage of 150 V at 20 kHz. (A) Fluorescence imaging of bacteria labelled with SYTO 9 (B) Fluorescence intensity of bacteria at different time. (C) Brightfield image showing bacteria trapping. All quantification data are presented as the mean $\pm$ SD of three independent experiments.

For the trapping experiments, 150 V at the frequency of 20 kHz was used. Figure 6.6 shows that a very high concentration of bacteria ($\sim 10^{10}$ CFU/ml) was achieved within 60 s.

6.3.3 Role of applied frequency

In contactless DEP (cDEP) devices, the voltage drop is highly dependent on the applied frequency due to the presence of a thin insulating dielectric gap between the medium and the electrodes, i.e. capacitive coupling. The voltage drop in the medium further plays an important role in the electric field strength and electric field gradient and thereby affects the DEP force as well as electroporation threshold. Figure 6.7

demonstrates the trapping of *E. coli* at various frequencies. Trapping performance was found to increase with an increase in the applied frequency. This is due to the dependence of the voltage drop across the medium on the applied frequency. At higher frequencies, the PDMS gap (i.e. capacitor) tends to conduct current as compared to lower frequencies, where the gap acts as an insulator. Figure 6.4D shows the increase in voltage-drop across the medium under various frequencies.

Figure 6.7: Trapping of *E. coli* for the applied voltage of 150 V at various frequencies: 1.25 kHz, 2.5 kHz, 5 kHz, 10 kHz and 20 kHz. (A) Green color represents *E. coli* fluorescently labelled with SYTO 9. (B) Brightfield image of *E. coli* at 1.25 kHz, 10 kHz and 20 kHz. (C) Fluorescence intensity of trapped *E. coli* at various frequencies. All quantification data are presented as the mean $\pm$ SD of three independent experiments.

Also, time scale analysis shows that the AC frequencies higher than 10 kHz suppress AC electric field induced EO and EP motions, which might affect DEP trapping in this work, as electric field were applied across the side electrodes. For AC frequency of 10 kHz, $\tau_{AC} = O\left(\frac{1}{2\pi f}\right) \sim O(10^{-5})s$. The EO flow relaxation time for 950 μm wide channel is obtained as $\tau_{EOF} = O\left(\frac{D_h^2 \rho}{\eta}\right) \sim O(10^{-2})s$ and the EP relaxation time for *E. coli* bacteria is obtained as $\tau_{EP} = O\left(\frac{R^2 \rho}{\eta}\right) \sim O(10^{-5})s$ (Lewpiriyawong et al. 2012).

6.3.4 Role of applied voltage

The frequency of 10 kHz was chosen to examine the effect of applied voltage, and the experiments were performed under various voltages: 50 V, 100 V, 150 V, 200 V and 250 V. Fluorescently labelled *E. coli* were used for the experiments. Figure 6.8 shows the results of the trapping under the various voltages. For 50 V and 100 V, no trapping effect was observed. A trapping effect was observed when the voltage was increased to 150 V. Subsequently, bacterial trapping increased with the increase in voltage. At higher voltages, the voltage drop across the medium also increased, resulting in higher electric field gradients and DEP force. Figure 6.8B shows the measured fluorescence intensity for the applied voltages at 10 kHz and 20 kHz. A higher bacterial concentration was achieved at lower voltage at higher frequencies.

Figure 6.8: Trapping of *E. coli* at an applied frequency of 10 kHz under various applied voltages. (A) Green colour represents bacteria fluorescently labelled with SYTO 9. (B) Fluorescence intensity of trapped *E. coli* at various voltages under 10 kHz and 20 kHz. All quantification data are presented as the mean $\pm$ SD of three independent experiments.

6.3.5 Inactivation test

In our cDEP device, bacteria are trapped in a high electric field using pDEP forces to enable cell electroporation. pDEP has been demonstrated experimentally and has been discussed in previous sections. This section demonstrates the inactivation of trapped bacteria and yeast cells. *E. coli* and yeast were used for the inactivation experiments. Figure 6.9 shows the inactivated *E. coli* and yeast cells at 300 V and 100 kHz. Both cells were labelled with SYTOX after trapping. However, the yeast cells were inactivated faster than the *E. coli* cells. This might have been due to the larger size of

the cells. Larger cells can be inactivated at lower electric fields as compared to smaller cells such as bacteria.

Figure 6.9: Demonstration of the inactivation of *E. coli* bacteria (top) and yeast cells (bottom) at an applied frequency of 100 kHz with the applied voltage of 300 V. The membrane of bacterial cells is impermeable to SYTOX dye, which can permeate the membrane of dead cells only. SYTOX emits a strong fluorescence signal upon binding with DNA

Figure 6.10 shows the quantitative measurement for the inactivation of *E. coli*. More than 3 log removal of *E. coli* was achieved. The bacterial cells were released after trapping for 5 min and were collected at the outlet for plate culturing.

Figure 6.10 Quantification *E. coli* and yeast cells inactivation at 300 V and 100 kHz by using plate culture method. All quantification data are presented as the mean $\pm$ SD of three independent experiments.

6.4 Summary

In this chapter, a contactless iDEP device is reported that can trap cells in a high-electric field region, using pDEP for inactivation. The device involved side channels filled with liquid metal and were isolated from the main channel by a thin PDMS gap. Both electrical modelling and numerical simulation were presented, which were in good agreement in determining the voltage-drop across the medium. The device was able to achieve high bacterial trapping as well as inactivation at a lower voltage than the conventional electroporation devices and the method can be used for both DEP and electroporation related applications.

Chapter 7: Conclusions and future works

7.1 Conclusions

Following the objectives of this thesis, extensive and rigorous experiments and numerical modelling were used to investigate cell electroporation using an iDEP device. First, a simple iDEP device was developed using circular micropillars to examine the inactivation of yeast cells and a parametric study was performed to determine how different parameters such as electric field and flow rate contributed to the inactivation performance. The experimental studies showed that the iDEP device had better inactivation performance compared to the micropillar-free device. Numerical modelling showed that electric field enhancement was achieved due to the presence of the insulating micropillars. The electric field could only electroporate cells if sufficient TMP was reached. From this study, it is believed that a low flow rate greatly supports the electroporation effect. This work could be very useful to those who are seeking continuous cell electroporation and can potentially be used in various biomedical and environmental applications such as genetic engineering, biochemistry and water treatment.

Second, a parametric study was conducted to examine the influence of various parameters such as micropillar shape, electric field type (AC or DC) and AC frequency on toe different bacterial types. It was found that a rhombus-shaped micropillar can create higher electric field regions compared to a circular one, which is due to the singularity point. This contributed to the higher bacterial inactivation effect for the rhombus-shaped micropillar device as compared to that of a circular one. It was also observed that a DC field was better compared to an AC field; however, the electrolysis effect and electrode corrosion are its limitations. Therefore, in this case, a low-

frequency AC field is better for bacterial electroporation. Use of very high frequencies reduced the electroporation performance of the device, as the induced TMP decreased with the increasing frequencies. Further, gram-positive bacteria were more electroporation resistant compared to gram-negative bacteria due to the presence of the thick peptidoglycan layer in gram-positive bacteria. This work can be useful to those who want to optimize electroporation in microchannels.

Our third study, as proof of concept, involved the use of insulating microbeads for enhancing the electric field to create strong local electric field regions. The device served as an efficient method of bacterial inactivation as compared to the reference microbead-free device. Inactivation performance was achieved in a very short amount of time and at much lower energy consumption compared to UV methods of bacterial inactivation. The parametric study performed during the experiments showed that it was difficult to inactivate gram-positive bacteria compared to gram-negative bacteria, which our microfluidic device had also demonstrated. Similarly, a unit cell model was developed that can be used for scaling purposes.

Finally, an iDEP device was presented that uses contactless electrodes using liquid metal for simultaneous DEP trapping and electroporation. Here, DEP trapping was used to trap cells in a high-electric field region, which can prolong the residence time, and can be fruitful in electroporation. Using side channels filled with liquid metal as electrodes greatly minimized the distance between two electrodes, which minimized the requirement for high voltage. Likewise, using contactless electrodes prevented the limitations of electrode-based devices. In our study, pDEP was achieved that allowed bacteria to be trapped in the high-electric field region, which were then electroporated. The electrical model was also presented to obtain a voltage drop in the medium, and the results were compared to that of numerical simulation. Both methods showed good

agreement in determining voltage drop across medium. This method can be used for various biomedical applications such as selective trapping and sorting of biological cells and particles. Our electrical model can be the base for other researchers who plan to use and optimize contactless DEP devices for various applications.

7.2 Recommendations for future works

In our work, there is still much room for improvement, which can be done to optimize the electroporation process. By optimizing the process, our methods can be useful for various areas of application such as genetic engineering, biochemistry, water treatment and the food industry. The following are a few recommendations for future work:

1. In this PhD study, inactivating microorganisms using irreversible electroporation was a prime focus. However, there are many other applications for irreversible electroporation, such as the extraction of genes or other subcellular organelles as well as proteins, which our method can achieve. iDEP devices can be developed for continuous extraction of intracellular organelles and is recommended as a future work. Likewise, the method can be optimized to explore applications based on reversible electroporation such as the delivery of genes, drugs or other molecules into the cell. The method can also be applied to recent breakthroughs such as gene editing (CRISPR-Cas9) and cell reprogramming (induced neurons).
2. In our work, experiments were conducted for sample with lower electrical conductivity. However, many electroporation related applications require the use of highly conductive buffer to preserve cell viability. Therefore, further experiments need to be performed with varying medium conductivities and pH. Similarly, numerical simulation was deployed for obtaining the distribution of the electric field, velocity field and temperature field. However, there are a few

methods for measuring the velocity (King et al. 2007) and temperature inside the microchannel accurately (Gallo-Villanueva et al. 2019b; Kunti et al. 2019).

Hence, it is recommended to measure the velocity and temperature and compare it with the numerical results for validation purpose.

3. In our work with contactless DEP, flow was provided using a syringe pump. Therefore, using a DC field for driving the flow of the suspension is recommended. Meanwhile, AC voltage can be applied using contactless electrodes to control DEP force (Lewpiriyawong et al. 2010, 2012). As a future work, this method can be applied to various applications of DEP. In addition, a rhombus-shaped micropillar can generate high electric field gradients, which can enhance DEP performance, and should be investigated.
4. Our approach for enhancing the electric field strength was based on the use of insulating structures such as micropillars or microbeads. However, the enhancement in the electric field was not very high and depended on the geometry and arrangement of the micropillars/microbeads. From our study in Chapter 3 and Chapter 4, gap between micropillars plays a very important role in enhancing the electric field. However, practically, the gap cannot be smaller than the size of cells to be electroporated. Therefore, extremely high electric field enhancement cannot be achieved. As a recommendation, developing and using very high-aspect ratio structures such as nanowires, nanorods or nanotubes is suggested. These structures work on the principle of the lightning rod effect and can significantly enhance the electric field strength as:

$$\frac{|\mathbf{E}_{tip}|}{|\mathbf{E}_0|} = \alpha \frac{l_{rod}}{d_{rod}}, |\mathbf{E}_0| = \frac{U}{d} \quad 7.1$$

where E_0 is the applied electric field, α is a constant and l_{rod} and d_{rod} are the nanostructure length and diameter, respectively. Nanowires, nanotubes and nanorods have extremely high aspect ratios (l/d), which provides sufficiently high field strength for electroporating cells at a much lower voltage. Therefore, developing these structures for electroporation purposes is recommended.

Figure 7.1: Fabrication and characterization of CuONWs. (A) Before air oxidation; (B) after air oxidation at 500°C. (C) Low magnification SEM image after CuONW formation (at $\times 55$). (D) high magnification SEM image after CuONW formation (at $\times 1500$)

CuONWs for developing electroporation-based devices for water treatment is currently being studied. Copper sponge is used because of its high electrical conductivity and high porosity. Figure 7.1 presents the CuONWs grown from copper sponge.

Appendix A. Induced transmembrane potential: Spherical cells

The cell membrane is a 5-nm thick phospholipid bilayer with embedded proteins and saccharides. The conductivity of cell membrane is in several orders lower than that of cytoplasm and external medium. Therefore, when the cell is exposed to an external electric field, the membrane acts as a capacitor and charges are induced across the membrane, giving rise to a potential, called induced transmembrane potential. Figure A.1 shows the induced transmembrane potential when placed inside an electric field.

Figure A.1: Induced transmembrane potential when the cell is placed in a uniform electric field.

During theoretical analysis, cells are usually considered spherical although real scenario can be different for different cells. Usually, the cell is assumed to be the cytoplasmic sphere bounded by a spherical cell of uniform thickness. The spatial and temporal distribution of electric field in and out of the cell membrane, when the cell is exposed to the homogeneous electric field, can be solved as

$$\nabla \cdot \left(\left(\sigma + \epsilon \frac{\partial}{\partial t} \right) \nabla \psi(x, y, z) \right) = 0 \quad \text{A.1}$$

where ψ is electric potential, σ is electrical conductivity and ϵ is dielectric constant. For steady-state analysis, time derivative component is assumed to be 0. Assuming no

change in electrical conductivities Equation A.1 can be simplified to Laplace's equation as

$$\nabla \bullet \nabla \psi(x, y, z) = \frac{\partial^2 \psi}{\partial x^2} + \frac{\partial^2 \psi}{\partial y^2} + \frac{\partial^2 \psi}{\partial z^2} = 0 \quad \text{A.2}$$

For spherical cells placed in an electric field, the spherical coordinate system is preferred to cartesian coordinate when solving for the spatial distribution of electric field which is given as

$$\nabla \bullet \nabla \psi(r, \theta, \phi) = \frac{1}{r^2} \left[\frac{\partial}{\partial r} \left(r^2 \frac{\partial \psi}{\partial r} \right) + \frac{1}{\sin \theta} \frac{\partial}{\partial \theta} \left(\sin \theta \frac{\partial \psi}{\partial \theta} \right) + \frac{1}{\sin^2 \theta} \frac{\partial^2 \psi}{\partial \phi^2} \right] = 0 \quad \text{A.3}$$

When a cell is aligned such that its center coincides with the origin of the coordinate system, and direction of electric field is parallel to the line traced by r when $\theta = 0^\circ$ and $\phi = 0^\circ$, then symmetry is obtained with respect to ϕ for constant values of r and θ . In that case, $\frac{\partial \psi}{\partial \phi} = 0$ and results in

$$\nabla \bullet \nabla \psi(r, \theta, \phi) = \nabla \bullet \nabla \psi(r, \theta) \quad \text{A.4}$$

Equation A.5 can be obtained in which ' ϕ ' is reduced.

$$\frac{1}{r^2} \left[\frac{\partial}{\partial r} \left(r^2 \frac{\partial \psi}{\partial r} \right) + \frac{1}{\sin \theta} \frac{\partial}{\partial \theta} \left(\sin \theta \frac{\partial \psi}{\partial \theta} \right) \right] = \frac{\partial^2 \psi}{\partial r^2} + \frac{2}{r} \frac{\partial \psi}{\partial r} + \frac{1}{r^2} \frac{\partial^2 \psi}{\partial \theta^2} + \frac{\cot \theta}{r^2} \frac{\partial \psi}{\partial \theta} = 0 \quad \text{A.5}$$

Equation A.5 can be solved using separation of variables in which electric potential ' ψ ' can be written as the product of functions of ' r ' and ' θ ' as

$$\psi(r, \theta) = G(r)H(\theta) \quad \text{A.6}$$

Combining Equations A.5 and A.6,

$$H(\theta) \left(\frac{\partial^2 G(r)}{\partial r^2} + \frac{2}{r} \frac{\partial G(r)}{\partial r} \right) + \frac{G(r)}{r^2} \left(\frac{\partial^2 H(\theta)}{\partial \theta^2} + \cot \theta \frac{\partial H(\theta)}{\partial \theta} \right) = 0 \quad \text{A.7}$$

Equation A.7 can be further arranged in which LHS consists only the function of ‘r’ whereas RHS consists only functions of ‘θ’ as

$$\frac{r^2}{G(r)} \left(\frac{\partial^2 G(r)}{\partial r^2} + \frac{2}{r} \frac{\partial G(r)}{\partial r} \right) = - \frac{1}{H(\theta)} \left(\frac{\partial^2 H(\theta)}{\partial \theta^2} + \cot \theta \frac{\partial H(\theta)}{\partial \theta} \right) \quad \text{A.8}$$

Equation A.8 can be separately written as two different second-order ordinary differential equations with constant k.

$$\begin{cases} r^2 G''(r) + 2rG'(r) - kG(r) = 0 \dots\dots\dots (a) \\ H''(\theta) + \cot \theta H'(\theta) + kH(\theta) = 0 \dots\dots\dots (b) \end{cases} \quad \text{A.9}$$

The general solution for Equation A.9(a) can be obtained as

$$G(r) = \begin{cases} C_1 r^{-1/2} \sin \left(\sqrt{\frac{-1}{4} - k} \log r \right) + C_2 r^{-1/2} \cos \left(\sqrt{\frac{-1}{4} - k} \log r \right), & k < -1/4 \\ \frac{C_1}{r} + C_2, & k = -1/4 \\ C_1 r^{-1/2(1-\sqrt{1+4k})} + C_2 r^{-1/2(1+\sqrt{1+4k})}, & k > -1/4 \end{cases} \quad \text{A.10}$$

where C_1 and C_2 are constants. Since, the value of electric field approaches homogenous condition with an increase in the value of ‘r’, $G(r)$ should be proportional to ‘r’ as $H(\theta)$ is not the function of ‘r’. It is to note that only when $k = 2$, above mentioned condition, is satisfied. Hence, Equation A.11 can be obtained:

$$G(r) = C_1 r + \frac{C_2}{r^2} \quad \text{A.11}$$

Moreover, the value of constant ‘k’ should be same for both equations in Equation A.9. Therefore, Equation A.9(b) can be solved more specifically as shown in Equation A.12 with constants C_3 and C_4 .

$$H(\theta) = C_3 \cos \theta + C_4 \left(1 - \cos \theta \log \sqrt{\frac{1 + \cos \theta}{1 - \cos \theta}} \right) \quad \text{A.12}$$

Singularities are obtained in Equation A.12. for the values of ‘ θ ’ at 0° and 180° which cannot be true because the value of $\psi(r, \theta)$ is finite for all values of ‘ r ’ and ‘ θ ’. Hence, $C_4=0$ and Equation A.13 is obtained.

$$H(\theta) = C_3 \cos \theta \quad \text{A.13}$$

From Equations A.6, A.11 and A.13,

$$\psi(r, \theta) = \left(Ar + \frac{B}{r^2} \right) \cos \theta \quad \text{A.14}$$

where ‘A’ and ‘B’ are arbitrary constants. Considering geometrical and electrical properties that consist of the cell membrane, cytoplasm, and the external medium, Equation A.15 can be written where subscripts ‘i’, ‘m’ and ‘e’ represents intracellular cytoplasm, membrane, and extracellular medium respectively. ‘R’ represents the radius of the cell and ‘d’ represents membrane thickness.

$$\psi(r, \theta) = \begin{cases} \psi_i(r, \theta) = (A_i r + B_i r^{-2}) \cos \theta, 0 \leq r \leq (R - d) \\ \psi_m(r, \theta) = (A_m r + B_m r^{-2}) \cos \theta, (R - d) \leq r \leq R \\ \psi_e(r, \theta) = (A_e r + B_e r^{-2}) \cos \theta, r \geq R \end{cases} \quad \text{A.15}$$

When $r = 0$, the potential value should be finite and hence, ‘ B_i ’ must be equal to 0. Moreover, the electric field far away from the cell must reach homogenous field ‘E’ and hence, for far field, it can be explicitly written as

$$\psi(r, \theta) = -Er \cos \theta \quad \text{A.16}$$

with $A_e = -E$. Remaining constants ‘ A_i ’, ‘ A_m ’, ‘ B_e ’ and ‘ B_m ’ can be obtained by applying following four boundary conditions. First two boundary conditions represent the continuity of electric potentials at two different interfaces and last two conditions

represent the continuity of electric current density at the same interfaces which are represented as in Equation A.17.

$$\begin{aligned}
\psi_i(R-d, \theta) &= \psi_m(R-d, \theta) \\
\psi_m(R, \theta) &= \psi_e(R, \theta) \\
\sigma_i \frac{\partial \psi_i(R, \theta)}{\partial r} \Big|_{r=R-d} &= \sigma_m \frac{\partial \psi_m(R, \theta)}{\partial r} \Big|_{r=R-d} \\
\sigma_m \frac{\partial \psi_m(R, \theta)}{\partial r} \Big|_{r=R} &= \sigma_e \frac{\partial \psi_e(R, \theta)}{\partial r} \Big|_{r=R}
\end{aligned} \tag{A.17}$$

Using Equation A.15 along the above-mentioned BCs, four different equations are obtained. Solving these four equations with known constants ‘A_e’ and ‘B_i’, following expressions for coefficients are obtained

$$\begin{aligned}
A_i &= \frac{-9ER^3 \sigma_e \sigma_m}{2R^3 (\sigma_m + 2\sigma_e)(\sigma_m + \frac{1}{2}\sigma_i) - 2(R-d)^3 (\sigma_e - \sigma_m)(\sigma_i - \sigma_m)} \\
A_m &= \frac{-3ER^3 \sigma_e (\sigma_i + 2\sigma_m)}{2R^3 (\sigma_m + 2\sigma_e)(\sigma_m + \frac{1}{2}\sigma_i) - 2(R-d)^3 (\sigma_e - \sigma_m)(\sigma_i - \sigma_m)} \\
B_m &= \frac{3ER^3 (R-d)^3 \sigma_e (\sigma_i - \sigma_m)}{2R^3 (\sigma_m + 2\sigma_e)(\sigma_m + \frac{1}{2}\sigma_i) - 2(R-d)^3 (\sigma_e - \sigma_m)(\sigma_i - \sigma_m)} \\
B_m &= \frac{ER^3 [R^3 (\sigma_m - \sigma_e)(2\sigma_m + \sigma_i) - (R-d)^3 (\sigma_m - \sigma_i)(2\sigma_m + \sigma_e)]}{2R^3 (\sigma_m + 2\sigma_e)(\sigma_m + \frac{1}{2}\sigma_i) - 2(R-d)^3 (\sigma_e - \sigma_m)(\sigma_i - \sigma_m)}
\end{aligned} \tag{A.18}$$

Finally, induced transmembrane potential can be obtained by the following expression:

$$\Delta \psi_m = \psi_m(R-d, \theta) - \psi_m(R, \theta) = f_s ER \cos \theta \tag{A.19}$$

where,

$$f_s = \frac{3\sigma_e [3dR^2\sigma_i + (3Rd^2 - d^3)(\sigma_m - \sigma_i)]}{2R^3(\sigma_m + 2\sigma_e)(\sigma_m + \frac{1}{2}\sigma_i) - 2(R-d)^3(\sigma_e - \sigma_m)(\sigma_i - \sigma_m)} \quad \text{A.20}$$

Considering cell membrane as a pure insulator, $\sigma_m = 0$, and f_s turns out to be $3/2$ resulting in famous steady state Schwan's equation presented in Equation A.21.

$$\Delta\psi_m = \frac{3}{2}ER\cos\theta \quad \text{A.21}$$

It is found that transmembrane potential is directly proportional to external electric field 'E', the radius of cell 'R' and the angle between the direction of electric field and the line joining center of the cell to point of interest.

Appendix B. Evolution of membrane potential

Cytoplasm is composed of a thick solution bounded by the cell membrane that comprises of several ions, water, and different proteins. The concentration of inorganic ions varies for different cells depending on their functions and physical and geometric conditions. Moreover, the concentration of different ions also varies with the cell type and their function. Microorganisms such as bacteria and yeast cells have high ionic concentrations of K^+ , Na^+ and Cl^- ions than mammalian cells. However, overall concentrations of inorganic ions in mammalian, yeast, and bacteria remain within same range. Table B1 presents the concentration of ions for different cells. Due to difference in the concentration of ions inside and outside of the cell membrane, chemical concentration gradient is induced. Additionally, an electrical gradient is also generated due to the differences in the charged species i.e. ions. As a result, a driving force is generated due to the differences in the chemical and electrical gradient which always tries to bring both forces into equilibrium. Nernst equation can be used to relate these two gradients. Under the equilibrium condition, Nernst equation is written as

$$E = \frac{RT}{zF} \ln \frac{[X_1]}{[X_2]} \quad B.1$$

where R is universal gas constant, T is the room temperature, z is the ion number and F is Faraday's constant. Above equation can also be written as

$$E_{mV} = \frac{-58}{z} \log \frac{[Conc_{in}]}{[Conc_{out}]} \quad B.2$$

Typically, this membrane potential is around -70 mV, inside being more negative. Similarly, dielectric properties of cytoplasm can be contributed by the presence of

charged metabolites and ions. Dielectric property of different cells is different; within the range of 50-150 whereas membrane has a dielectric constant in the range of 2-15.

Table B1: Ionic concentrations (in mM) in sea water, a bacterial and yeast cell, inside a mammalian cell and in the blood. More BNIDs used to construct table: 104083, 107487, 110745, 110754.

ion conc. (mM)	sea water	<i>E. coli</i>	<i>S. cerevisiae</i>	mammalian cell (heart or RBC)	blood plasma	BNID
K ⁺	≈10	30-300	300	100	4	104049
Na ⁺	≈500	10	30	10	100-200	104050
Mg ²⁺	≈50	30-100 (bound); 0.01-1 (free)	50	10 (bound) 0.5 (free)	1	104983, 100770, 101953
Ca ²⁺	≈10	3 (bound); 100 nM (free)	2 (bound)	10-100 nM (free)	2	100130, 110746, 111366
Cl ⁻	≈500	10-200 media dependent		5-100	100	105409, 110744
BNID	106594	105926, 107033, 107114, 111425	107752	103966, 107187	105409	

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