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**PAPER BASED MICROFLUIDICS FOR RAPID
PRECONCENTRATION OF MICROPOLLUTANTS BY ION
PRECONCENTRATION**

PERERA ADHIKARIGE TANIYA KAUSHALYA
INTERDISCIPLINARY GRADUATE SCHOOL
Nanyang Environment and Water Research Institute
Advanced Environmental Biotechnology Centre

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PRECONCENTRATION**

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**Interdisciplinary Graduate School
Nanyang Environmental and Water Research Institute
Advance Environmental Biotechnology Centre**

A thesis submitted to the Nanyang Technological University in
partial fulfillment of the requirement for the degree of
Doctor of Philosophy

2020

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Statement of Originality

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January 16, 2020

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Date

Prof. Yang Chun, Charles

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Authorship Attribution Statement

This thesis contains material from two papers published in the following peer-reviewed journals where I was the first author.

Chapter 3 is published as Perera, A. T. K., Phan, Dinh.-Tuan., Pudasaini, Sanam., Liu, Yu. and Yang, Chun. (2020). Enhanced sample pre-concentration by ion concentration polarization on a paraffin coated converging microfluidic paper based analytical platform, *Biomicrofluidics*, 14: 014103. DOI: 10.1063/1.5133946.

The contributions of the co-authors are as follows:

- Prof Chun Yang provided the initial project direction and edited the manuscript drafts.
- I prepared the manuscript drafts. The manuscript was revised by Dr Dinh-Tuan Phan, Prof Yu Liu and Prof Chun Yang.
- I co-designed the study with Dr. Dinh-Tuan Phan and performed all the laboratory work at the School of Mechanical and Aerospace Engineering. I also analyzed the data.
- All research works including sample preparation, characterization, testing and analysis were done by me.
- Sanam Pudasaini helped in preparation of 3D schematics.

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- Prof Chun Yang provided the initial project direction and edited the manuscript drafts.

- I prepared the manuscript drafts. The manuscript was revised by Dr Dinh-Tuan Phan, Prof Yu Liu and Prof Chun Yang.
- I designed the study and performed all the laboratory work at the School of Mechanical and Aerospace Engineering. I also analyzed the data.
- All research works including sample preparation, experimental characterization, testing and analysis were done by me.
- Sanam Pudasaini and Syed Shaheer Uddin Ahmed helped in cell culture and figure drawings.

6th January 2020

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Date

Perera Adhikarige Taniy Kaushalya

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3. Pudasaini, Sanam., **Perera, A.T.K.**, Das, D., Ng, S.H. and Yang, Chun. (2019), Continuous flow microfluidic cell inactivation with the use of insulating micropillars for multiple electroporation zones. *Electrophoresis*, 40: 2522-2529. doi:10.1002/elps.201900150
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LIST OF ABBREVIATIONS

ICP	- Ion concentration polarisation
μPAD	- Microfluidic paper-based analytical device
ICP-MS	- Inductively coupled plasma – mass spectrometry
MS	- Mass spectrometry
AAS	- Atomic absorption spectrometry
R-FS	- X-ray fluorescence spectroscopy
LC-MS	- Liquid chromatography-mass spectrometry
HPLC	- High performance liquid chromatography
PCR	- Polymerase chain reaction
DNA	- Deoxyribose Nucleic Acid
ITP	- Isotachophoresis
FASS	- Field amplified sample stacking
PDMS	- Polydimethylsiloxane
CF	- Concentration factor
FITC-BSA	- Fluorescein isothiocyanate- Bovine serum albumin
CEM	- cationic exchange membrane
AEM	- anionic exchange membrane
<i>E. coli</i>	- <i>Escherichia coli</i>
EDL	- Electric double layer
DC	- Direct current
EOF	- Electroosmotic flow
SCL	- Space charge layer
DL	- diffusion layer
CPL	- Concentration polarisation layer
CIMD	- Current induced membrane discharge

EK	-	Electrokinetic
NT	-	Electroneutrality
1D	-	One dimensional
2D	-	Two dimensional
PMMA	-	Polymethyl methacrylate
HF	-	Hydrogen fluoride
PEM	-	Polymer electrolyte membrane
μ - FFE	-	Miniaturizing free flow electrophoresis
3D	-	Three dimensional
AKD	-	Alkyl ketene dimer
LOD	-	Limit of detection
USEPA	-	United states environmental protection agency
CFU	-	Colony forming units
ATCC	-	American type culture collection

LIST OF SYMBOLS

Greek symbols

ψ_s	-	Surface potential
ψ_d	-	Stern layer potential
ζ	-	Zeta potential
ψ	-	Potential based on surface charge
ε_o	-	Permittivity of vacuum
ε_r	-	Relative permittivity of medium
κ	-	Debye – Huckel parameter
λ_D	-	Debye length
σ_s	-	Surface charge density
ρ_e	-	Space charge density
σ_d	-	Charge density at Stern plane
P_{fix}	-	Fix charge density of nanoporous structure
ρ	-	Density of solution
η	-	Dynamic viscosity
ε_P	-	Porosity of membrane
$\mu_{\pm}$	-	Electrophoretic mobility of cation or anion
δ	-	Diffusion layer thickness
β^{∞}	-	Ratio between maximum and initial concentration
κ	-	Permeability of paper
γ	-	Surface tension
θ	-	Contact angle
σ	-	Electric conductivity
K	-	Permittivity
μ_{eo}	-	Electroosmotic mobility
μ_{ep}	-	Electrophoretic mobility

φ	-	Porosity
ρ_{paper}	-	Density of paper
ρ_{fibre}	-	Density of cellulose fibres

English symbols

e	-	Elementary charge
n_i^∞	-	Bulk number concentration
z_i	-	Valance of i^{th} ionic species
k_B	-	Boltzmann constant
T	-	Absolute temperature
N	-	Number of ionic species
U	-	Fluid velocity
J_i	-	Flux density of ion species i
c_i	-	Concentration of ion species i
D_i	-	Diffusion coefficient of ion species i
N_A	-	Avogadro's number
$J^\pm$	-	Flux of cations or anions
$\vec{u}$	-	Slip velocity
$\mathbf{E}$	-	Electric field strength
v	-	Convective velocity
d_{ec}	-	Vortex height
w	-	Width of channel
h	-	Height of channel
U_{HP}	-	Average velocity for Hagen-Poiseuille flow
$C_{EK/NT}^\infty$	-	Maximum concentration for electrokinetic/ electroneutrality limit
V_{cm}	-	Cross membrane potential
Q	-	volumetric flow rate

ΔP	-	Pressure drop
r	-	Average pore radius of the porous media
t	-	Time
I	-	Electric current
U	-	Electroosmotic flow velocity
q	-	Effective charge of particle
r	-	Stokes radius of the particle
u	-	Linear velocity of particle
V_p	-	Pore volume
V_b	-	Bulk volume
A	-	Area of the device covered by the solution
T	-	Thickness of paper channel
Da	-	Dalton

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ABSTRACT

The contamination of water bodies heavily contributes to the water scarcity experienced globally. Hence, water is continuously monitored under various quality chapters, such as physical, chemical, or biological quality, as contaminants of water vary in a wider range. The contaminants can be organic compounds, inorganic compounds, or even pathogenic microorganisms. Some contaminants may not present at levels that are detectable by conventional monitoring techniques; this is often the case for micropollutants such as heavy metal ions, antibiotic residues, pathogens, or industrial chemicals. These contaminants pose risk to human/animal life and the environment at lower concentrations, therefore it is of great importance to monitor them continuously. Nevertheless, the presence of these contaminants in potable and recreational water in tracer amounts often requires preconcentration prior to analysis, and current methods—such as solid/liquid phase extraction, followed by mass spectrometry, liquid chromatography-mass spectrometry, and so forth, or cell culturing—are time-consuming, expensive, and complex. In this scenario, the use of electrokinetic phenomena such as ion concentration polarisation (ICP) for the preconcentration of charged particles/ions in a less complicated microfluidic system with reduced specific requirements becomes a viable option for the rapid and easy detection of these micropollutants. ICP is an ionic transport phenomenon that enables the preconcentration of low-concentrated analytes, whereas microfluidics enables the use of minimal sample amounts, thus facilitating the detection process. This study focused on developing a microfluidic paper-based analytical

device (μ PAD) to implement ICP and study the application of ICP on a μ PAD to detect various types of micropollutants, identifying its characteristics and optimising the process. First, this thesis presents a novel, rapid, inexpensive, and convenient method for fabricating μ PAD, showing its feasibility to detect low concentrations of analytes through the enhanced preconcentration of samples in a shorter time. Based on the developed method, μ PAD was fabricated and studied experimentally for the preconcentration of *Escherichia coli* through ICP, addressing the issues encountered in conventional detection methods, such as the consumption of a significant amount of time. The research proceeded to study the performance of ICP on a μ PAD in detecting heavy metal ions, where ICP is characterised for such smaller ions of heavy metals. Finally, the performance of ICP in the detection of antibiotics, complex molecules that tend to change their polarity based on media, is reported. The research studied various classes of micropollutants, providing insight into the use of ICP and its characteristics based on the type of analyte from a broader perspective.

CHAPTER 1 INTRODUCTION

1.1 Overview

Water is an essential component of life for all living beings. Even though the earth is called “blue planet,” the available amount of fresh water for human and other living beings is approximately 2.5% of the total water on Earth, and the greatest proportion of this is stored in non-accessible forms, such as ground water and ice [1, 2]. Theoretically, water on Earth circulates through hydrological cycles, and the amount of water does not diminish. Still, the scarcity of water is a reality experienced globally, since the replenishment of water is largely dependent on the replenishment rate and the temporal and spatial distribution of water. Moreover, consumptive usage, pollution of water, and climate change contribute heavily to the water scarcity around the globe, threatening not only human and animal life but also economic growth in a global scenario [1, 3, 4]. Hence, securing limited water resources has become a major responsibility from a global perspective.

A fundamental requirement for securing water resources is continuous monitoring of water quality, including the pollution of these water bodies, as water quality continues to deteriorate day by day. Bartram and Ballance define water quality as “long-term, standardized measurement and observation of the aquatic environment in order to define status and trends” [5]. For these standardized measurements, water is monitored under various quality chapters, such as physical, chemical, or biological, as the contamination of water can occur due to various forms of contaminants [6, 7]. For an example, these can be

either organic contaminants or inorganic contaminants. Further, depending on the contaminant release rate, the amount of contaminant added to the water or capacity of the water body, the concentration of targeted contaminants may be in the detectable range or below the detectable range of conventional equipment used in monitoring. This is often the case with monitoring the quality of potable and recreational waters for a variety of micropollutants, such as heavy metal ions, residual antibiotics and their metabolites, pathogens, or industrial chemicals. These are present in potable and recreational water in tracer amounts, rendering it difficult to monitor their presence employing conventional methods, and often require pre-treatment prior to analysis. Nevertheless, monitoring such contaminants for their concentrations is of greater importance, since these substances often pose a severe threat to human health and aquatic life and, in a broader picture, support water scarcity, that is, most of the heavy metals dissolved into water are highly toxic at tracer concentrations and are non-degradable and cause bio accumulation [8]. Some of the contaminants, such as some antibiotics or their residues, have not yet been fully studied for their presence and impact on human or animal health, as identifiable quantities have not been found during analysis [9]. Further, cases of pathogenic bacterial outbreaks causing water-borne diseases, public beach closures, and so forth have been reported [10, 11].

Micropollutants such as heavy metal ions and antibiotic residues are currently studied using solid-liquid extraction, liquid-liquid extraction, and liquid membrane extraction, based on adsorption and extraction fundamentals [12-15], followed by analytical techniques, such as inductively coupled plasma-mass spectrometry (ICP-MS), mass spectrometry (MS), atomic absorption spectrometry (AAS)—electrothermal,

graphite furnace—X-ray fluorescence spectroscopy (R-FS) [16-18], liquid chromatography-mass spectrometry (LC-MS), and high-performance liquid chromatography (HPLC)[19-21]. These methods are complicated, time-consuming, and expensive and require trained personnel and extensive sample preparation and purification prior to analysis [22, 23]. As for the pathogenic microorganisms, their detection is mainly achieved through cell culturing or molecule-based cell amplifying methods that multiply the cell population to a detectable level, starting from a very small concentration. These methods are also highly time-consuming (typically 24 – 48 hours), require trained personnel, and are not suitable where the concentration of pathogens can considerably vary within a day's time [10, 24-26]. In addition to these, polymerase chain reaction (PCR) is available for pathogen detection based on deoxyribonucleic acid (DNA) amplification. Nevertheless, the need to maintain critical environmental conditions and the need for extensive sample preparation limit its applicability [24, 27, 28]. Therefore, these methodologies are mostly suitable for analytical laboratory purposes rather than for rapid, in-field measurements.

Within this context, the use of detection techniques such as electrochemical detection [8, 26, 29, 30], colorimetric detection [31, 32], electrochemiluminescence [33], and fluorescent detection [34-37] for monitoring micropollutants rapidly in a minimally technical background/environment has captured researchers' attention, as these techniques enable miniaturization of detecting probes/platforms to a greater extent [8, 18, 22]. Some of these detection techniques are used alongside analytical techniques such as LC-MS or MS [36, 38]. Methods such as electrochemical detection also provide a concentration effect to the analyte, improving the sensitivity of the device [29, 39].

The reported literature shows that prior to most detection techniques, analytes are pre-treated to concentrate them into a detectable range. Further, efforts in the miniaturization of analytical devices suggest the application of pre-concentration techniques in miniaturized environments. Such methods include enzyme-based signal enhancement, microfluidic PCR used in particle and DNA amplification [40], and evaporation-based concentration, which is applicable to concentrate any target in few folds [41]. The stacking methods—such as isotachopheresis (ITP), sweeping, and field-amplified sample stacking (FASS)—enable few orders of magnitude in concentration factor but require the use of specific buffers in operation [41-43]. Electrokinetic phenomena, such as ion concentration polarization (ICP), enable pre-concentration of charged particles/ions in a less complicated system with reduced specific requirements [41, 44].

ICP is a phenomenon that is induced near a perm selective nanoporous membrane under the presence of a direct current (DC) electric field for the purpose of concentrating a targeted analyte. Since perm selective membranes allow only one type of ion to pass through (ions with an opposite charge to the membrane charge, counter-ions), an ion imbalance forms in the system, creating ion enrichment and ion depletion zones at either side of the membrane. This leads to the concentration of charged particles/ions at the edge of the depletion zone (for a cationic exchange membrane—e.g., nafion—this occurs at the anodic side, and vice versa), allowing this region to be used as a concentration band for the concentration of diluted sample solutions of charged species. The phenomenon can also be induced near a micro nano junction where a nanoporous membrane is replaced by a nanofluidic channel [45-49]. Following efforts to implement ICP on miniaturized

devices, in 2015, Phan et al. developed a continuous flow droplet-based concentrator on a polydimethylsiloxane (PDMS) glass substrate, showing a 100-fold sample concentration of a sample with droplet size ranging from 20 – 25 μm [47]. Later, in 2017, Ouyang et al. showed a concentration factor (CF) of around 95 for Alexa Fluor 488 dye on a device made of silicon [50], and in 2018, Marczak et al. reported a 15-fold CF for exosomes within 10 mins in a polycarbonate channel [51]. Conventional microfluidic chip designing, however, comprises complex and time-consuming procedures that hinder the advantages of using them in sample analysis, and the materials used in fabrication are relatively expensive [24, 50, 52].

In this context, microfluidic paper-based analytical devices (μPAD) have captured researchers' attention as a viable substitution for conventional microfluidic analytical devices. These devices are made using paper as the basic substrate, and hydrophobicity is introduced in numerous ways, where necessary. μPAD s offer advantages such as low cost for material and fabrication, disposability, and passive capillary wetting [53-55]. Recently, several researchers have paid attention to the effect of ICP for sample concentration on a μPAD . In 2016, Yeh et al. achieved a CF of 944 within 30 mins for fluorescein salt at the ion enrichment zone, using ICP [56]. Recently, Gao et al. reported achieving a CF of 10 for fluorescein isothiocyanate labelled bovine serum albumin (FITC-BSA) using cationic exchange membrane (CEM) and a CF of 50 for rhodamine 6G using anionic exchange membrane (AEM) [57]; this was followed by Han et al., who reported concentration factors of not more than 40 for human serum [58]. Still, the technology is quite new and indicates the enormous space available for research in terms of developing novel

fabrication methods for robust devices and characterization of ICP on μ PADs for various applications.

1.2 Scope and Objectives

The objective of this thesis is to explore the possibility of concentrating various sample solutions on a microfluidic paper-based platform implementing ICP. To accomplish this goal, a novel fabrication method for microfluidic paper-based devices is developed, which enables rapid and easy fabrication of these platforms. The work performed here then analyses the effect on sample concentration from the perspective of the main factors involved in ICP. The scope of this thesis is established under the following objectives:

- Develop novel, less time-consuming, more cost-effective, and more flexible fabrication methods for the fabrication of μ PAD and study the influence of factors including heat, pressure, and temperature on the process of fabrication to optimize these parameters to fabricate a μ PAD with the abovementioned qualities and study the fabricated device for the influences of geometric configuration, electroosmotic flow, applied electric field, and evaporation of sample solutions during the implementation of ICP.
- Develop a μ PAD, using the novel fabrication method introduced, for the preconcentration of pathogenic bacteria found in water. The device is investigated for the effect of total ion concentration in cell suspension media on ICP and the fluorescence intensity observed. The effect of the electroosmotic flow of a cell suspension on ICP is also studied.

- Develop a μ PAD for the preconcentration of heavy metal ions and study the factors—including applied electric field, diffusivity of ions, size of ions, affinity of ions towards perm selective membrane, and choose perm selective membrane—that affect the preconcentration of these ions.
- Develop a μ PAD to enable preconcentration of aqueous antibiotic residues, utilizing the property of functionalizing the molecules based on the environmental conditions. The factors of study are suitable fluorescent detecting probes for antibiotics without affecting the charged nature of the molecule, the binding characteristics of fluorescent probes and antibiotics, the residual charge of the complex formed, and the applied electric field.

The μ PAD is fabricated utilizing Whatman No. 1 chromatography paper, paraffin films, and off-shelf perm selective membranes (e.g. Nafion®), using a novel method of direct cutting and oven heating. This fabrication method is used throughout the study, and μ PADs with different configurations are developed to achieve the preconcentration of various analytes and demonstrate that the developed method is flexible, rapid, and convenient and enables the fabrication of low-cost μ PADs with different geometric configurations for various applications.

1.3 Outline of the Thesis

This thesis consists of seven chapters including this introductory chapter to the research project.

Chapter 2 provides a comprehensive review of relevant literature. The chapter begins with a state-of-the-art survey of the literature on transport phenomena in microfluidic/nanofluidic channels, and special attention is given to the theoretical and conceptual background of ICP, a phenomenon that occurs near a micro-nanojunction, and its applications. A detailed review of the literature on the integration of nanoporous junctions into microfluidic platforms is provided; this is followed by the final major section, which contains a comprehensive review of the literature on μ PADs. This section reviews the use of paper as a microfluidic channel substrate, types of μ PADs, transport phenomena in microfluidic paper channels, currently available fabrication methods for μ PADs, highlighting the drawbacks and potential for the development of new methods, and μ PADs reported for the implementation of ICP. The limitations of applications of ICP in μ PADs are discussed, and the ample room available for extending the domains of utilizing ICP as a preconcentration technique is highlighted.

Chapter 3 presents a detailed study of developing a novel, rapid, low-cost, easy-to-use, and flexible fabrication method for μ PADs. The chapter provides a comprehensive study of the applicability of the device for the preconcentration of diluted sample solutions and the effects of factors such as the applied electric field, hydrophobic barrier substances, and geometric configuration on the concentration factor observed during ICP.

To investigate the applicability of ICP in a μ PAD for the effective preconcentration of pathogens in environmental waters, a study on the preconcentration of *Escherichia coli* through ICP on a simple μ PAD is presented in **Chapter 4**. The study highlights the limitations associated with currently available conventional methods for the

preconcentration and detection of these pathogens and addresses the bottlenecks identified in introducing ICP as a viable, rapid preconcentration and detection method for *E. coli*. The effect factors, such as total ion concentration in cell suspension media, on ICP and fluorescent intensity are also studied here.

Chapter 5 reports on the application of ICP for the detection of aqueous heavy metal ions through preconcentration of heavy metal ions on a μ PAD. A detailed study on the effect of perm selective membrane, size, diffusivity, and affinity of heavy metal ions on the implementation of ICP is presented in this chapter.

Chapter 6 addresses the use of ICP for the preconcentration of aqueous antibiotic residues, another class of micropollutants, which are neutral in nature but can be functionalized, depending on environmental conditions, such as the pH of the media. The chapter elaborates on suitable fluorescent-detecting probes for the ICP of antibiotics, potential binding characteristics of fluorescent probes and antibiotics, and the charged nature of the complex formed on the concentration of the analyte using ICP.

Finally, **Chapter 7** concludes the thesis by providing a summary of the findings of this thesis, alongside proposed recommendations for future work to study the phenomenon further.

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CHAPTER 2 LITERATURE REVIEW

2.1 Transport Phenomena in Microfluidic/ Nanofluidic Channels

2.1.1 Electrostatic Effects

2.1.1.1 *Electric Double Layer*

The surface of objects immersed in electrolytic fluids shows the ability to become charged through the absorption of ions on the surface, the ionization/dissociation of functional groups on the object surface, or various other causes. The ions that charge the surface are of similar signs and render the surface negatively or positively charged. The ions that possess an opposite charge to that of the surface charge are called counter-ions, while the ions that bear a similar charge to the surface charge are called co-ions. The counter-ions in the solution are attracted to the surface due to Coulomb force in an effort to neutralize the surface charge. Due to thermal motion, however, these ions form a second layer bound loosely around the surface, making a diffuse layer. In simple terms, these two parallel layers of ions of opposite charge make the electric double layer (EDL). Still, in reality, EDL takes a complex form. The inner boundary of EDL is defined by Otto–Stern, in which the thickness is around one hydrated ion radius. This is known as the Stern plane. Formation of the second layer leads to the creation of an ion cloud around the object that mostly comprises counter-ions. The ions in this cloud, known as the Gouy–Chapman layer, are quite mobile and diffusive. From one to two hydrated ion radius thickness from the surface is the inner mobile portion of the Gouy–Chapman layer, which is known as the shear plane. Together, the Stern layer and the Gouy–Chapman layer form the Debye layer, which is characterized by double-layer thickness. The potential reduces

from the surface potential ψ_s to ψ_d at the Stern plane and zero when moving further away from the surface. The potential at the shear plane is defined as zeta (ζ) potential, where no-slip conditions are assumed to apply. EDL thickness typically falls within a small number of nanometres of the length and tends to increase as the bulk electrolyte is diluted [59-61].

2.1.1.2 Perm Selectivity of Nanochannels/ Nanoporous Structures

Considering the formation of EDL in a channel or a porous structure and typical EDL length, inside a large enough channel or porous structure, such as in micro scale, Debye length becomes comparatively shorter, to the scale of micro channel or micro pore. Therefore, the neighbouring ion concentration of EDL is similar to that of the bulk electrolyte. The electric potential at the centre of the channel or pore is almost zero. By contrast, as the channel or pore structure becomes narrower into nano scale, EDL from the wall overlaps inside the channel/porous structure. It is the electric potential at the centre of the channel/porous structure that is comparably high and of the same sign as the surface potential that leads to this EDL overlapping. In such a scenario, enrichment of the counter-ions occurs inside the nanochannel/nanoporous structure while co-ions are expelled to the bulk electrolyte from the nanochannel/nanopore interface. This effect is known as the ion enrichment- exclusion effect (EEE). The transport of counter-ions through nano structures occurs by diffusion, whereas electrostatic repulsion obstructs co-ions from entering the nano structure at the interface. Hence, nanochannel/nanoporous structures are able to show perm selectivity or selective transport of one type of ion over

the other due to this EEE. Such is the basic phenomenon of the perm selectivity of nanoporous membranes and structures [59, 61, 62].

2.1.1.3 Potential Distribution in EDL

Figure 2.1: (A) Schematic of Gouy-Chapman-Stern model for surface-electrolyte interface (electric double layer). Shown below is the electrostatic potential distribution ψ within EDL versus the distance z normal to the surface. Zeta potential ζ is measured at slip plane [63]. (B) EDL overlapping as channel pore size reduces from micrometre to nanometre scale. (i) Distribution of ion concentration and electrostatic potential inside a microchannel. (ii) Distribution of ion concentration and electrostatic potential inside a nanochannel [48].

The potential distribution inside the nanochannel/nanoporous structure can be explained by the one-dimensional Poisson equation, modified by Boltzmann distribution as

$$\nabla^2 \psi = \frac{d^2 \psi}{dx^2} = -\frac{e}{\varepsilon_0 \varepsilon_r} \sum n_i^\infty z_i \exp \left[-\frac{z_i e \psi(x)}{k_B T} \right] \quad (2.1)$$

where ψ is the electric potential dependant on the surface charge, e is the elementary charge, which is 1.602×10^{-19} C, ε_0 is the permittivity of the vacuum, ε_r is the relative permittivity of the medium, n_i^∞ is the bulk number concentration, z_i is the valance of the i^{th} ionic species, k_B is the Boltzmann constant of 1.381×10^{-23} J K⁻¹, and T is the absolute temperature [59, 61, 62].

2.1.1.4 Debye – Huckel Approximation

For the cases where ψ is sufficiently low, that is, $z_i \psi_i < 25.6$ mV at 25° C, the Poisson Boltzmann equation (equation 2.1) can be linearized into the following form, which is known as Debye – Huckel approximation.

$$\nabla^2 \psi = \frac{d^2 \psi}{dx^2} = \kappa^2 \psi(x) \quad (2.2)$$

Here, κ refers to the Debye – Huckel parameter, defined as follows:

$$\kappa = \left(\frac{e^2}{\varepsilon_0 \varepsilon_r k_B T} \sum_{i=1}^N z_i^2 n_i^\infty \right)^{1/2} \quad (2.3)$$

where N refers to the number of ionic species. The reciprocal of κ ($1/\kappa$) is defined as the Debye length (λ_D), which is the characteristic length of EDL. Since the potential ($\psi(x)$) decays when moving further from the surface, equation 2.2 can be solved to obtain the following:

$$\psi(x) = \psi_s \exp(-\kappa x) \quad (2.4)$$

For a symmetrical electrolyte (e.g. KCl), equation 2.2 can be elaborated using the following form:

$$\tanh\left[\frac{z_i e \psi(x)}{4k_B T}\right] = \tanh\left[\frac{z_i e \psi_s}{4k_B T}\right] \exp(-\kappa x) \quad (2.5)$$

Here, $e\psi/k_B T$ is the dimensionless potential. This model is known as the Gouy–Chapman model [59, 61, 64, 65].

2.1.1.5 Surface Charge Density and Zeta Potential

As the system is scaled down from macro to micro to nano scale, the surface-area-to-volume ratio increases, and this increment greatly influences the behaviour of the electrolytes within the nano-scaled system. Surface charge of the object is gained through either of the ways mentioned in section 2.2.1.1. The magnitude and the type of charge vary depending on the acidic or basic nature of the electrolyte, the types of ions present in the system, and so forth. Surface charge density (σ_s) for an object immersed in an electrolyte can be expressed as follows:

$$\sigma_s = \int_0^{\infty} \rho_e(x) dx \quad (2.6)$$

where ρ_e is space charge density [59, 64].

This equation can be extended to include zeta potential (ζ), with the aid of the charge density in the diffuse layer, which relates to the charge density at the stern plane (σ_d). This is because in theory, ζ is commonly considered identical to ψ_d [59, 66, 67].

Hence, the equation takes the following form:

$$\sigma_d = \left(2\varepsilon_0\varepsilon_r k_B T \sum n_i^\infty \left[\exp\left(-\frac{z_i e \zeta}{k_B T} \right) - 1 \right] \right)^{1/2} dx \quad (2.7)$$

2.1.2 Electrokinetic Phenomena

Electrokinetics is the study of the movement of particles, ions, or molecules under an electric field applied in parallel to the path of transport (i.e., nanochannel or nanoporous structure). Electrokinetic effects are raised by the presence of EDL at the solid-liquid interface, and four distinct phenomena can be identified under electrokinetic effects. Electroosmosis is the transport of an electrolyte solution under an applied electric field relative to a stationary charged surface. Electrophoresis refers to the movement of charged particles/ions relative to the surrounding medium under a uniform electric field, whereas dielectrophoresis refers to the movement that occurs in a non-uniform electric field. In addition to these, streaming potential is the phenomenon of creating an electric field under the forced movement of a liquid relative to a charged surface, while sedimentation potential defines the generation of an electric field when charged species

are transported relative to the surrounding media [61, 62, 68]. Considering the transport of charged species in a sufficiently diluted electrolyte through nano-scaled structures, the phenomenon is described using a complex interaction between several parameters. The parameters considered are the ion concentration of the species i (c_i), fluid velocity (U), and electric potential (ψ) in the system. This interaction can theoretically be explained by the governing equations for electrokinetic and transport phenomena within the system, which are the Poisson equation for the relation between electric potential and ionic concentration in the solution, the Nernst–Planck equation, which governs the flux of ion species i and tracer molecules within the system, and the Navier–Stokes equation explaining laminar incompressible fluid flow within the nanochannel/nanoporous structure [62, 69, 70].

2.1.2.1 Poisson – Boltzmann equation

The potential distribution of ions/charged species inside the nanochannel/nanopore is explained by the Poisson equation, as follows:

$$\nabla^2 \psi = \frac{d^2 \psi}{dx^2} = -\frac{\rho_e(x)}{\epsilon_0 \epsilon_r} \quad (2.8)$$

where

$$\rho_e = \sum_{i=1}^N z_i e n_i \quad (2.9)$$

and n_i refers to the ionic number concentration of i^{th} ionic species. The Poisson equation is modified with the volumetric fix charge density of the nanochannel/nanoporous structure

(ρ_{fix}) term when it is applied for the nano structure domain, which leads the Poisson equation to take the following form:

$$\nabla^2 \psi = -\frac{1}{\epsilon_0 \epsilon_r} \sum_{i=1}^N z_i e n_i + \rho_{fix} \quad (2.10)$$

Meanwhile, the Boltzmann distribution explains the probability of finding an ion in a given location within the nanochannel as follows:

$$n_i = n_i^\infty \exp\left(-\frac{z_i e \psi}{k_B T}\right) \quad (2.11)$$

in which $z_i e \psi$ refers to the work required to bring an ion from infinity ($\psi = 0$) to the position x , having a potential ψ .

2.1.2.2 Nernst – Plank Equation

The flux of ionic species i and tracer molecules is governed by the Nernst–Plank equation illustrating the balance between diffusion, electro-migration, and convection. The equation takes the following form:

$$\frac{\partial c_i}{\partial t} = -\nabla \cdot J_i \quad (2.12)$$

$$J_i = -D_i c_i - \frac{D_i e z_i \nabla \psi}{k_B T} c_i + U c_i \quad (2.13)$$

where J_i is the flux density of ion species i , c_i is the concentration of ion species i , and D_i is the diffusion coefficient of ion species i . The three terms to the right side of

equation 2.13 represent the flux of ions by diffusion due to the concentration gradient, the flux of ions from electro-migration, and the flux of ions through convective ion transport, respectively. This convective ion transport may be supported by the pressure gradient, mechanical forces, or external force fields.

Combinations of the Nernst-Planck and Poisson equations are known as Poisson-Nernst-Planck (PNP) equations. These non-linear PNP equations do not possess analytical solutions and are solved numerically for the systems, where applicable.

Under a low Reynold's number, the convective term of equation 2.13 can be neglected and rearranged.

$$J_i = -D_i \left(n_i + \frac{z_i e n_i}{k_B T} \nabla \psi \right) \quad (2.14)$$

$$c_i = n_i N_A \quad (2.15)$$

where N_A is the Avogadro's number.

When the system is at a steady state (SS), the continuity equation for the flux suggests that $\nabla \cdot J_i = 0$. Therefore, the SS Nernst-Planck equation for low Reynold's numbers is as follows:

$$\nabla \cdot \left(n_i + \frac{z_i e n_i}{k_B T} \nabla \psi \right) = 0 \quad (2.16)$$

2.1.2.3 Navier Stokes Equations

The mass conservation within a system with steady incompressible fluid flow is given by the continuity equation.

$$\nabla \cdot U = 0 \quad (2.17)$$

Furthermore, for the motion of a laminar incompressible flow with viscosity, the Navier-Stokes equation illustrates the conservation of fluid momentum:

$$\rho \left(\frac{\partial U}{\partial t} + (U \cdot \nabla) U \right) = -\nabla P + \eta \nabla^2 U - \rho_e \nabla \psi \quad (2.18)$$

where ρ is the density of the solution and η is the dynamic viscosity. Here, the term $-\rho_e \nabla \psi$ refers to the electrical body force induced during electrokinetic flow. Like in the Nernst–Planck equation at low Reynold’s numbers, laminar flow in micro/nano channels is dominated by viscosity. Hence, the convective term can be excluded, and equation 2.18 can be reduced into the Stokes equation, as given below.

$$\nabla P = \eta \nabla^2 U - \rho_e \nabla \psi \quad (2.19)$$

2.1.2.4 Brinkman Equation

With the involvement of a porous medium (filter paper/Nafion), however, the flow inside porous media in the absence of external hydrostatic pressure can be expressed using the Brinkman equation, which considers the effect of permeability (κ) and porosity (ε_p), instead of the Navier-Stokes equation. The Brinkman equation takes the following form:

$$\frac{\rho}{\varepsilon_p} \frac{\partial U}{\partial t} + \frac{\eta}{\kappa} U = \frac{\eta}{\varepsilon_p} \nabla^2 U - \eta \rho_e \nabla \psi \quad (2.20)$$

Here, $0 < \varepsilon_p < 1$ denotes the porosity of the membrane or porous media involved, and n is the correction factor for double-layer overlapping in the membrane.

Like the PNP equations, these coupled partial differential equations (PDEs) including PNP equations are solved numerically for the phenomena where these equations govern the species in the system.

2.2 Ion Concentration Polarization

2.2.1 Phenomenon of ICP

2.2.1.1 Phenomenon Description

Ion concentration polarization is one of the interesting phenomena observed in the presence of nanochannels/nanoporous membranes making a micro-nano junction. Even though the phenomenon has been observed for over 100 years, the scientific background of this phenomenon has been difficult to understand. This is due to the multi-physics that are involved in the multi-scale nature of the system. Within the system are physical phenomena such as ionic transport, non-linear fluid flow, and dynamic electric fields that are coupled together. Further, as the system comprises micro-nano interfaces, these physical phenomena occur in a multi-scale environment that ranges from micro- to nano-scale [69, 70]. The first experimental study of ICP was reported by Pu et al. in 2004 [71]. The phenomenon has attracted increasing attention due to its characteristics, and research efforts studying its theoretical basis, operational behaviour, applications of the

phenomenon, and methods of developing micro-nano platforms have been reported over the years.

ICP is an ionic transport phenomenon that occurs near a perm selective nanoporous membrane/nanochannel, which is induced by the formation of an ion enrichment zone and an ion depletion zone under the presence of a DC electric field. The dimensions of these nanoporous structures of membranes or nanochannels are typically comparable to the EDL thickness that forms within the system. Hence, inside these nanopores or nanochannels, EDLs from the walls overlap, giving rise to an ion enrichment-exclusion effect. This leads counter-ions to prevail within the nanopore/nanochannel, expelling co-ions from the micro-nano interface due to the perm selectivity of the nanopore/nanochannel. The total flux of ions/charged species through the perm selective nanopore/nanochannel is supported by both diffusion due to the concentration gradient (∇c) created within the system through the ion enrichment effect and the applied electric field. Total flux can be given as follows:

$$J^{\pm} = J_D^{\pm} + J_{EP}^{\pm} = -D_{\pm} \nabla c^{\pm} - c^{\pm} \mu_{\pm} E \quad (2.21)$$

where $D_{\pm}$, $c^{\pm}$, and $\mu_{\pm}$ denote the diffusivity of the cation or anion, the concentration of the cation or anion, and the electrophoretic mobility of the cation or anion, respectively. As a result of this, an ion enrichment zone and an ion depletion zone are created at either side of the micro-nano junction to maintain the electroneutrality within the system. This leads all charged species to accumulate in a narrow region at the edge of the depletion zone, giving rise to a concentration band, which is also called the sample plug, that can be used to pre-concentrate dilute samples of charged species of analytes [72-74].

Figure 2.2: Schematic of ion concentration polarization phenomenon at a micro-nano interface. (A) Creation of ion depletion and ion enrichment zones in a microchannel- nanochannel interface. (B) Creation of ion depletion and ion enrichment zones where a cationic exchange membrane (CEM) acts as a nanoporous junction.

2.2.1.2 Theoretical Limits of ICP

The implementation of ICP involves Multiphysics in a multi-scale environment, as explained in the previous section. With the involvement of Multiphysics, such phenomena as non-linear electrokinetic flow over limiting current and micro vortices are observed within the system, which introduce limits on the operation of the system. These behaviours are studied mostly in terms of their components and rarely as a whole.

It is typical to observe amplified local electric fields in the depletion zone at micro-nano junctions where ICP occurs. This leads to the formation of vortex-like flow, which is commonly known as micro vortices at the face of the depletion zone. This flow differs from equilibrium electroosmotic flow (EOF), as it moves faster and introduces a second type of EOF to the system due to this vortex instability. Therefore, the counter-ion flux into the nanochannel/nanoporous membrane increases and changes the equilibrium of the system [72, 75, 76]. Further, an over-limiting current is observed in the system at higher voltages that surpass a critical voltage. This current exceeds the limiting current defined for the system to implement ICP and increases with applied voltage. This over-

limiting current is explained by several theories, such as electroconvection, water splitting at high voltages, giving rise to ions and electrostatic effects. The theory of electroconvection, however, was rejected by Anderson et al. for a number of reasons. They cited the differences in the scale of the system (Macro to micro-nano scale), the potential for electrostatic influences in forming vortices, the heterogeneous nature of the micro-nano junction, and so forth [77, 78]. The theory of water splitting was also excluded by Rubinstein et al., as water dissociation alone is insufficient to raise the current to such a magnitude. Rubinstein et al. conducted several studies on this and proposed that this over-limiting current is a result of secondary EOF and vortex instability. They also suggested that vortices form at the nanochannel/nanoporous membrane entrance once voltages exceed the critical voltage for performing ICP. They presented a mathematical derivation for two-dimensional non-equilibrium electroosmotic slip velocity through a flat ion exchange membrane [79-81]. This suggests that the slip velocity ($\vec{u}$) is a quadratic function of the applied electric field ($\mathbf{E}$) and the relationship is as follows:

$$\vec{u}_s = |E|^2 / 8 \times (\partial^2 c / \partial x \partial y) / (\partial c / \partial y) \quad (2.22)$$

Here, x and y refer to the length parallel and perpendicular to the nanochannel/nanoporous membrane, respectively.

Kim et al. also studied the non-linear electrokinetic mixing in nanoporous structures in a microfluidic platform in conjunction with the over-limiting current to propose a relationship between these two phenomena. Their argument was that the formation of strong vortices leads to the destruction of the concentration gradient within

the ion depletion zone. They scaled the diffusion layer thickness (δ) as $\delta \sim (xD/v)^{0.5}$, where v is convective velocity, and suggested $\delta \sim 1/E$. According to their study, they concluded that fast convection at the nanochannel/nanoporous membrane is a mechanism to explain over-limiting current and that this convection can be restrained through the reduction of micro-channel thickness [72].

Later, Yossifon and Chang studied the formation of the depletion layer and vortex instability in the over-limiting region. Their work followed Rubinstein and Shtilman's theory on the occurrence of the extended polarized layer called the space charge layer (SCL) at high voltages between the EDL and the electroneutral diffusion layer (DL). This is supposed to withhold the over-limiting current. The combination of these three layers is identified as the concentration polarization layer (CPL), and the depletion layer formed during ICP closely represents this CPL, as it is a combination of the SCL and the DL. In an effort to experimentally confirm Rubinstein's instability, they created an intermittent depletion zone, with the aid of an alternating current (AC) electric field. Their results demonstrated that the CPL is determined by instability and follows the linear relationship for the width of the cell versus the depletion layer length at varying frequencies. Further, they stated that depletion layer thickness (δ) does not follow Dukhin's prediction of $\delta \propto 1/E$ [82] for voltage dependence; rather, it varies as a quadratic function of electric field E [76].

In 2012, Andersen et al. focused on the effects of chemical reactions (i.e., water dissociation) and charge regulation on the over-limiting current. They suggested a method named "current-induced membrane discharge" (CIMD), which is based on water splitting

and membrane (de)protonation for membranes with a greater thickness than the EDL. They demonstrated that CMID can result in the over-limiting current. The current is partly supported by co-ions in the bulk electrolyte, where ions by water render electrodialysis ineffective. CMID is involved in pH control and current-assisted ion exchange in the system. Further, it restrains the non-equilibrium space charge, which guides the system towards electroosmotic instability [77].

In another attempt, Chang et al. studied the effect of microhydrodynamics on ion flux through nanochannels/nanoporous structures when over-limiting current occurs. Their study examined the formation of microvortices and over-limiting current from a hydrodynamical perspective so that modifications to the system, such as asymmetric entrances that will form various polarized zones, can change the non-linear electrokinetic behaviour of the system. This in turn allows systems to be used as nanofluidic diodes, relay, and so forth to generate an ion current where necessary. An interesting fact about these is that the new systems can also be explained hydrodynamically [75].

Further, Kwak et al. investigated the phenomenon of electroconvective flow in ICP for pressure-driven shear flow. The group studied vortex generation from various perspectives—such as direction, height, and advection—with combined experimental and numerical studies. Based on their observations, under a pressure-driven flow, vortex height (d_{ec}) scales as follows:

$$\frac{d_{ec}}{w} \sim C \left(\frac{\phi^2}{U_{HP}} \right)^{1/3} \quad C = \left[\frac{\varepsilon}{64\mu w^3} \left(\frac{2wh}{w+h} \right)^2 \right]^{1/3} \quad (2.23)$$

where w and h refer to the width and height of the channel and U_{HP} is average flow velocity under Hagen-Poiseuille flow. The resulting scaling was a complete deviation from scaling for the flow under the diffusion drift model, and the results showed electroconvection as a major means for over-limiting current occurring specially in large scale systems with ion exchange membranes [78].

Although many studies have been conducted to elucidate the complexities of various aspects of ICP—such as non-linear flow, vortex formation, and so on—studies on these coupled phenomena were lacking until recently. Recently, Ouyang et al. addressed this problem by establishing the first theoretical model for concentration based on ICP. They defined two limits for the concentration factors observed under ICP as the electrokinetic (*EK*) limit and the electroneutrality (*NT*) limit. The *EK* limit is defined when the interested particle concentration is greatly lower than the concentration of buffer ions and buffer ions dominate the system in terms of fluid flow and electric field. Here, the maximum concentration (C^{∞}_{EK}) is observed based on the balance between the electrophoretic, diffusive, and convective flow of particles. The *NT* limit is defined for the case where particles (negatively or positively charged) act as majority carriers in the ion enrichment zone and respective ions in the buffer are near zero. The maximum concentration (C^{∞}_{NT}) is based on the particle amount required to neutralize the counter-ion carriers in the buffer solution. Their one-dimensional analysis for concentration factors (β^{∞}), defined as the ration between the maximum concentration and the initial concentration ($C_{3,0}$), provided the following expressions.

For the *EK* limit, considering symmetric binary electrolytes,

$$\beta_{EK}^{\infty} = \frac{C_{EK}^{\infty}}{C_{3,0}} \approx a^{-a} b^b (a-b)^{(a-b)} \cdot e^{(a-b) \cdot Pe} \quad (2.24)$$

where suffixes ₁, ₂, and ₃ refer to cations in buffer, anions in buffer, and particles, respectively, and $a = D_{12}/D_3$, $b = Z_3/Z_2$, where D_{12} is the diffusivity of the symmetric binary electrolyte.

In the *NT* limit for binary electrolytes, β_{NT}^{∞} is illustrated as follows:

$$\beta_{NT}^{\infty} = \frac{C_{NT}^{\infty}}{C_{3,0}} \approx \frac{Z_1}{Z_3} \cdot \frac{\mu_1/\mu_2 + 1}{\mu_1/\mu_3 + 1} \cdot \frac{C_{1,0}}{C_{3,0}} \quad (2.25)$$

Based on these derivations and experimental results, they stated that $\ln \beta_{EK}^{\infty} \propto V_{cm}$ and the enrichment effect is bounded by the *NT* limit where a transition from the *EK* limit to the *NT* limit is seen at sufficiently high cross-membrane potentials (V_{cm}). Further, the group explained that the concentration factor observed for the *EK* limit is lower at higher buffer concentrations due to limitations of membrane saturation [69].

2.2.2 Integrated Nanoporous Junction

There are various ways of integrating a micro-nano junction for the implementation of ICP. The method can be either fabrication of micro and nanochannel structure or integration of a nanoporous material, such as nafion, into the microchannel system [74].

2.2.2.1 *Micro-Nanojunction Based on Nanochannels*

The fabrication of a well-defined nanochannel structure for fluid transport is possible using microfabrication techniques. The available processes for the fabrication of nanochannels can be categorized as top-down or bottom-up approaches. For nanochannels of the smallest dimensions that are well below 100 nm, well-established top-down approaches are recommended, and the recommended method of fabrication is to design feed flow in perpendicular to the nanochannel, which reduces the risk of fouling inside the nanochannel [63]. Considering the dimensions of channels and other required properties, Pyrex is widely used as the substrate material for the fabrication of nanochannels. These properties are hydrophilicity, which allows for capillary wicking for channel filling, transparency, rigidity, and non-conductive nature [63, 83, 84]. The high dissolution rate of Pyrex in water (10^{-11} cm/s at room temperature), however, engenders swelling and loosening of the nanochannel structure over time and the formation of silica gel [85, 86]. To minimize these drawbacks, performing surface modification for the nanochannel is recommended [87, 88]. In addition to minimizing these drawbacks, modification can be used to introduce foreign properties into nanochannels, rendering them smart [89]. In addition to this, a variety of silica—such as silicon nitride, polysilicon, and other polymers or metals—are used for the fabrication of nanochannels [90].

Some of the methods available for the fabrication of such nanochannels are wet and dry etching [83, 90], nanoimprint lithography [91, 92], sacrificial layer methods [93, 94], high-energy beam techniques [95-97], and bonding and sealing methods [84, 98].

Both one-dimensional (1D) and two-dimensional (2D) nanochannels can be fabricated through etching. Two-dimensional nanochannels of smaller diameters, such as 20 nm, are fabricated. The channel is first etched to the silicon substrate through reactive ion etching and photolithography, followed by bonding onto a glass or silicon substrate [83]. For glass substrates, buffered oxide etching is used for the nanomachining process [63]. For 1D channels, etching techniques such as bulk nanomachining, surface nanomachining, bond and mould nanomachining, or wet anisotropic etching can be used [90] .

Nanoimprint lithography was developed by Chou and Krauss as an inexpensive, high-throughput nonconventional method for the fabrication of 2D nanochannels. The method is capable of constructing channels with smooth and perpendicular side walls to the substrate material [99]. Resist materials, such as polymethyl methacrylate (PMMA) [91, 99] and silicon dioxide [92], are used during fabrication.

A nanometre-thick sacrificial layer is used in the sacrificial layer method of nanochannel fabrication for the realization of nanochannels on the substrate. Later, this layer is removed by means of etching. Nanofluidic filters [100] and nanofluidic transistors [93, 101] are constructed, using silicon dioxide as the sacrificial layer. In addition, silicon nitride is used as a capping material for metal nanowires in constructing longer nanochannels [94].

High-energy beam techniques, such as ion beam milling, are used for the direct construction of nanochannels of several tens of nanometres into a substrate. This method

is employed in the fabrication of 1D nanochannels in materials such as glass [95], fused silica [97], or quartz [63, 96].

In bonding and sealing, encapsulation of the fabricated nanochannels is performed. Techniques such as anodic bonding, glass fusion bonding, or chemically activated bonding are employed during this process. The two wafers to bond are first stacked and heated up to a temperature range of 300 - 500°C to render them electrically conductive; a voltage in the range of 100 - 1500 V is then applied [84]. In chemically activated bonding, two wafers are soaked with solutions such as sulfuric acid for the required time and later exposed to chemical wafers such as hydrofluoric acid (HF). As soon as these wafers are removed from the steam, they can be directly bonded together [98].

2.2.2.2 Micro-Nanojunction Based on Nanoporous Material

A micro-nanojunction can also be fabricated by incorporating nanoporous material into a microchannel platform. In most of the published work, Nafion® has been used as a nanoporous material, either in the form of resin or in the form of off-shelf membrane, for the fabrication of a micro-nanojunction. The use of such resins of nanoporous material enables the formation of nanoporous layers to be convenient and rapid. In a series of work by Han's group, Nafion® resin was used to create the nanojunction that serves as a cation selective membrane [102-107]. Han's group patterned a submicron-thick Nafion® resin onto a glass substrate. Their results showed a well-defined strip of Nafion® on the glass substrate. The need for PDMS master mould to define the flow of Nafion® and the application of negative pressure to suck out the extra resin, however, lead to inconsistent thickness of the deposited Nafion® strip [102]. Further, the bonding between the glass

substrate and Nafion® is so weak that it can be easily peeled off of the surface, and the finishing of the patterned strip is not smooth. A similar method has been used in several works by R.J Yang's group and other researchers [41, 44, 46, 108-112]. Alternatively, Nafion® resin has been used to form a nanoporous layer in a range of studies conducted by researchers applying other methods. Such efforts include the use of micro printing [113], the self-sealed junction method [114], and the dripping of Nafion® resin directly onto the channel, mainly where paper-based microfluidic channels are used [56, 115-118]. These methods also exhibit their own advantages, such as the ease of generating a nanoporous layer, and disadvantages, such as the difficulty of controlling the dispersive nature of Nafion® resin in the channel.

In an effort to address these issues, Phan et al. introduced the use of off-shelf Nafion® membrane as the nanoporous layer in the creation of a micro-nano junction [119] and used it in several studies [47, 120]. The membrane can be used for both PDMS micro channels and paper-based microfluidic channels.

Aside from Nafion®, several other ion exchange membranes have also been used by researchers in an effort to create micro-nano junctions. In a study of concentration polarization, Neosepta CMX containing sulfonic acid groups and Neosepta AMX with active components of quaternary ammonium groups were used as cation exchange membranes (CEM) and anion exchange membranes (AEM), respectively [121]. Neosepta CMX membrane was also used as a CEM to study ICP in a later study [80]. Further, zwitterionic polymer has been used for the laser patterning of nanoporous membranes in a microchip for the electrophoretic concentration of proteins [122]. Moreover, ionic

hydrogel 2-hydroxyethylmethacrylate (HEMA)-acrylic acid (AA) and dimethylaminoethyl methacrylate (DMAEMA) as cation and anion selective hydrogels have also been used to fabricate nanoporous junctions [123]. In addition, in a study of electrokinetic trapping, a negatively charged hydrophilic polymer known as 2-acrylamido-2-methyl-1-propanesulfonic acid was employed to create a nanoporous membrane [124]. The use of hydrogel for the formation of nanoporous membranes other than Nafion® has also been reported [108]. Moreover, Fumasep® FTAM-E and FTCM-E were used as AEM and CEM [78] in a study of shear flow by ICP for the formation of a nanojunction. Later on, a modification to Nafion® was introduced, in which it was mixed with graphene oxide (GO) to fabricate a nanoporous membrane [125]. In recent studies, the use of CEMs that are negatively charged with organosulfonate groups for the isolation and preconcentration of exosomes using ICP [51] and a homogeneous CEM and an AEM named AMI-7001 to implement ICP on a paper-based microfluidic device other than Nafion® [57] has been reported.

2.2.3 Fabrication of Nafion® Based Nanoporous Junction

Nafion® is a synthetic polymeric membrane that is the first of a class known as ionomers, where ionic properties are introduced to the polymer. The membrane is synthesized through copolymerization of tetrafluoroethylene, which acts as the backbone of this linear polymer, with perfluorovinyl ether that contains a sulfonate functional group at the end [126-129]. The negatively charged hydrophilic sulfonate group (SO_3^-) exhibits strong cation exchange capabilities (Fig 2.3 A). With these functional groups that are incompatible with one another connected to a fluorocarbon backbone, Nafion® assumes a

“cluster network model” in which sulfonic acid head groups are arranged in an inverted micelle structure imbedded in a continuous fluorocarbon backbone. These clusters are interconnected with narrow channels and determine the transport properties of the membrane (Fig 2.3 B). This is essentially the cation selectivity of the membrane, which is governed by the inverted micelle structure formed by the sulfonic acid groups [127, 129-131]. In addition, microscopic studies have concluded that Nafion contains ionic channels in nanoscale that are aligned in parallel in a polymer matrix (Fig 2.3 C) and that hydration of the membrane leads to the swelling of these channels perpendicular to the channel and enables easy conduction of protons through the capillary structure [132].

Further, this combination of a fluorocarbon backbone and ionic groups offers unique properties to this material. The membrane shows extreme resistance to chemicals, such that only strong alkali metals such as sodium are able to attack the membrane under general operation conditions.

Moreover, Nafion® exhibits remarkable thermal and mechanical stability. The operation under extreme temperature conditions, however, differs for different models of Nafion®. For instance, the Nafion® 115 membrane starts to exhibit changes at a temperature of around 180°C, whereas another type of Nafion® with an equivalent weight of 1000 g showed melting at around 275°C [131, 133, 134]. Considering these properties, the membrane is extensively used in fuel cells as polymer electrolyte membrane (PEM) for proton conduction and also as an acid catalyst [129, 132-134]. Even though other PEMs have been developed in the industry in recent years, the perfluorosulfonic membrane developed in 1962 by Walther G. Grot at Du Pont, which is commercially

identified as Nafion®, is still considered a state-of-the-art membrane for PEMs [127, 128, 132].

Figure 2.3: Characterization of Nafion®. (A) Structure of Nafion® showing (SO_3^-) sulfonic group [134]. (B) Cluster network model of Nafion® with SO_3^- arranged in an inverted micelle imbedded in a fluorocarbon backbone. The clusters are connected by narrow channels [130], copyright 2004, American Chemical Society. (C) Representation of the hydrophilic and hydrophobic domains of the Nafion® membrane. Hydrophilic SO_3^- groups combine with water to form hydrophilic domains (blue), and these domains connect at a higher hydration rate, forming an ionic channel network [132]. (D) Current sensing atomic force microscopy (CSAFM) images for a fresh Nafion 212 membrane (right) and an aged Nafion 212 membrane (left) with proton current profiles on the sample along the line scan [132], copyright 2010, American Chemical Society.

2.2.3.1 Use of Nafion® Resin

Fabrication of a micro-nano junction by incorporating Nafion® resin is a common practice and is performed by following several methods. The most common of these methods is the patterning of a submicron-thick layer of Nafion® on glass or the substrate of interest (Fig 2.4 A). Here, a PDMS microchannel is reversibly bonded to the glass substrate to define the flow path of the Nafion® perfluorinated resin. Once the introduction of a microliter of Nafion® resin has been completed, positive pressure or negative pressure is applied at the inlet of the channel or outlet of the channel, respectively, to fill up the channel completely. Afterwards, this glass substrate is placed in an oven or on a hotplate for the desired amount of time, at the desired temperature, for the curing of the Nafion® resin. The PDMS mould is then removed, and a thin strip of Nafion® on a glass substrate is obtained. As PDMS is flexible, the microchannels fabricated with PDMS can easily conform to the shape of the Nafion® strip and be bonded using oxygen plasma treatment [102-105, 108-111].

One of the other methods for fabricating a nano junction using Nafion® resin is micro contact printing or micro stamping (Fig 2.4 A). Here, a PDMS microstamp is used to extract Nafion® from a well, and it is printed on the glass substrate and cured. The PDMS microchannel is then bonded with the surface-patterned glass substrate through an oxygen plasma treatment. One of the drawbacks of this method is that upon bonding, the PDMS channel and the printed membrane may become sealed due to deformation of the PDMS channel. This method, however, allows for creating an array of preconcentrators [113].

The self-sealed method is another method for creating nano junctions using Nafion® resin (Fig 2.4 B). In this method, the PDMS microchannel is first fabricated using lithographic methods, and sample loading holes are punched. The microchannels are then cut through mechanically, using razor blades, to a depth that passes beyond the channel height. This gap is then opened by bending the PDMS chip, and Nafion® is introduced into this gap. Nafion® quickly fills up the gap and a part of the microchannel due to capillary wicking. Following this, curing is performed at 95 °C for 10 minutes, and any residual Nafion® in and on the microchannel is removed through tapping. Here, the flexible nature of PDMS helps in sealing the Nafion junction between PDMS walls [114].

Figure 2.4: Fabrication of Nafion® based nanoporous junction. (A) Micro flow patterning (left) and micro stamping (right) for the fabrication of a Nafion® strip on the substrate [102]. (B) Self-sealed method of fabricating a Nafion junction in a micro channel [114]. (C) Use of off-shelf Nafion membranes to produce nanoporous junctions [119].

Dripping Nafion® resin directly onto the microchannel is another well-practised method of fabricating micro-nano junctions, especially in paper-based microfluidic

devices. During this process, Nafion® resin is directly pipetted onto the paper channel where necessary in microlitres (0.5 – 6 μ L) and allowed to air dry or oven dry for the desired period of time. In some cases, the hydration of the Nafion® is accomplished by dipping the device in deionized water, or rinsing the Nafion® is accomplished, using tris buffer, to achieve improved performance [56, 115-118].

2.2.3.2 *Use of Off-Shelf Nafion® Membranes*

The methods discussed above for the fabrication of nano junctions using Nafion® resin exhibit some disadvantages, such as the need for a PDMS master mould, a fabrication process consisting of several steps, and dispersion of the resin out of the boundaries of the area of interest. Hence, the use of off-shelf Nafion® membranes as nano junctions was introduced by Phan et. al. [119]. In this method, a cleaned glass slide is first spin-coated with PDMS (around 5 ml per glass slide) at the required rate so that the PDMS thickness would match the thickness of the off-shelf Nafion® membrane to be used (e.g. for Nafion® 212 of 50 μ m thick, a spin speed of 500 rpm for 10 s and 1450 rpm for 30 s is used.) The thin Nafion® strip is then placed on the spin-coated glass slide and kept stationary on a flat surface for the reflow of PDMS overnight. A secondary spin-coating step can be performed if required to ensure a flat surface on the glass slide (Fig 2.4 C). Finally, these glass slides are cured in an oven for the desired amount of time, at the desired temperature [47]. The method can also be applied to paper-based microfluidic devices. Here, the Nafion® membrane is cut into the desired shape and placed on or below the paper channel at the required position, and it is sealed by means of encasing or taping [120].

2.2.4 Applications of ICP

The phenomenon of ICP can be employed in several practical applications, such as separation of charged species, mixing, and sample preconcentration.

2.2.4.1 *Separation*

Separation of ions/charged species from a bulk phase is often required in purification applications. Use of ICP for the separation of charged species was first manifested by Kim et. al. [107] for sea water desalination. During implementation of ICP, the seawater splits into two streams, named the desalted stream and brine or the concentrated stream, as it passes over an ion selective membrane, such as Nafion®. As ICP repels the ions or other charged species from the membrane, forming a depletion zone, rather than attracting them towards the nanoporous membrane, the use of ICP avoids the problem of membrane fouling found in conventional desalination processes. The concentrated stream can be diverted from the main flow direction, allowing desalted water to pass through the main channel passively. The application of the phenomenon, however, was small-scale, and the reproducibility was low. Hence, starting from this, Han's group continued to study ICP for desalination from many perspectives, such as improving the efficiency of the process and the use of the phenomenon for scaled-up desalination. Later, they showed that unipolar ICP offers enhanced salt removal and reduced power consumption (by 50%) in desalination, as compared to the conventional electrodialysis process [73]. Further, they showed that ICP is suitable for the purification of highly concentrated brine solutions through incorporating multi-stage ICP platforms and can achieve around 70% salt removal [135]. Another study on desalination was conducted by

MacDonald et. al. [136] to investigate its scalability. The device fabricated was multiplexed three-dimensionally and scaling up showed a 3-fold improvement in functional density.

Figure 2.5: Employing ICP for separation: (A) Schematic of introducing ICP for desalination [107], copyright 2010, Springer Nature. (B) Schematic of using ICP for continuous separation of particles [112], copyright Springer Nature. (C) Schematic for extraction of neutral compounds in an ionic micellar phase using ICP [137], copyright 2019, RSC. (D) Schematic showing desalted and concentrated streams in ED and ICP platforms [73], copyright 2016, Springer Nature.

Counter to this application, Jeon et al. [112] used ICP for the separation of two types of polymeric beads based on the differences in their electrophoretic mobilities. The concept employed was to use the effect of the strong electric field in the depletion region for the repulsion of different kinds of particles and separate them into focused flow paths. This method involves no use of internal electrodes and, hence, is cost effective, and the operation is bubble-free, unlike in the conventional methods of continuous electrophoretic separation, such as miniaturizing free flow electrophoresis (μ - FFE). A recent study by Berzina et al. reported on the extraction of neutral compounds using ICP. Berzina et al. used the characteristic of forming micelles by neutral compounds—in this case, hydrocarbons at the presence of a surfactant—to create an ionic micellar phase that enabled the performance of ICP [137].

2.2.4.2 *Mixing*

Mixing can be realized through the adoption of electroconvection effects that occur within the concentration polarization zone. It is a well-known fact that there appear to be local non-linear electrokinetic responses (a second kind of EOF) adjacent to the ion depletion zone, giving rise to strong convective mixing at sufficiently high voltages. This is attributable to the formation of non-equilibrium space charge layers by ion depletion within the system [70, 72, 79]. Inspired by these characteristics observed in ICP, Kim et al. [138] developed a micro/nanofluidic mixer device for the active mixing of solvents. The device was fabricated through two-step reactive ion etching of silicon, following a simple design, where nanochannels act as the vortex generator to mix the solvents residing within the microchannel. Later on, Jannig et al. [139] developed a nanofluidic mixer utilizing a

nanoporous Nafion® membrane in a polymeric device made of PMMA. The device enabled the use of pressure-driven flow.

Figure 2.6: Schematics of micro/nanofluidic mixers utilizing ICP: (A) Device fabricated by two-step reactive ion etching. Microchannel and nanochannel depth are 4 μm and 60 nm, respectively [138]. (B) Schematic for the concept of pressure-driven flow micro/nanofluidic mixer utilizing Nafion® as nanoporous membrane. Mixing channel turns and crosses the inlet channels at another plate, where they are separated at the junction, using a contact slip and Nafion membrane. Under the electric field across the inlet and outlet, ICP is implemented at the nano junction, and at a higher voltage, strong vortices lead the mixing zone towards the entrance of the mixing channel [139].

Kwak et al. studied this electroconvective flow under a pressure-driven flow theoretically and demonstrated that these vortices can be scaled with respect to the applied electric field and the pressure-driven flow velocity [78]. Another effort to employ the second type of EOF behaviour in ICP for the purpose of mixing is a micro/nanofluidic mixture developed by Chiu et al. [140], incorporating a Nafion® membrane with a PDMS micro-channel structure.

2.2.4.3 Preconcentration

Concentrating tracer analytes prior to detection is of critical importance to bringing their concentrations up to the detection ranges specified for analytical platforms.

Electrokinetic trapping based on ICP offers preconcentration of ions/charged species on less complicated platforms with reduced special conditions [41, 44]. The phenomenon of ICP possesses unique features, in that, for example, the molecules of interest are independent of the hydrophobicity of the binding characteristics and the use of simple electrodes enables the implementation of ICP [102, 120]. Hence, ICP can be incorporated into a wide range of microfluidic devices for the concentration of diluted sample solutions. As described in section 2.2.1.1, concentration of diluted samples can be obtained at the edge of the depletion zone as a concentration band. Over the years, several studies have been conducted in which ICP has been used as the preconcentration technique. In 2005, Wang et al. developed a nanofluidic filter using standard photolithography and etching to enable ICP. The device comprises two microchannels connected by a nanochannel. One of the microchannels acts as the buffer channel, whereas the other serves as the sample channel. The device enables concentration factors around $10^6 - 10^8$ [141]. Later, Lee et al. demonstrated the use of ICP in a multiplexed PDMS device with a surface-patterned Nafion membrane. The parallel array of channels enabled the increment of the sample volume for signal enhancement [113]. Scraff et al. demonstrated ICP for the concentration of proteins in a radial microfluidic device, where concentration occurred at a central region of the chamber, which was analysed at the upper level [142]. Later, Choi et al. demonstrated the concentration of two different samples, using ICP, where the electrophoretic properties of the samples were used in the creation of two separate bands. A pneumatic valve system was integrated to collect the concentrated sample plugs selectively [45]. Marczak et al. also demonstrated the isolated concentration of exosomes with the aid of ICP in an agarose-gel-filled micro-nano channel system. Electrophoretic

properties were used as the isolation technique [51]. Recently, Ouyang et al. developed a device named nanofluidic crystal, which enabled them to perform reconfigurable ICP for the concentration of biomolecules. The group used nanoparticles to form a nanochannel network in a microchannel and created the micro-nanojunction [50].

Figure 2.7: (A) Schematic depicting concentration mechanism in a micro-nanochannel integrated system [141], copyright 2005, American Chemical Society. (B) Multiplexed PDMS microfluidic platform for implementation of ICP with an array of 10 single preconcentrators [113]. (C) A magnified cross-section of a radial microfluidic chamber where concentration occurs while ICP is implemented [142]. (D) Microscopic image of the device for selective preconcentration of different samples [45], copyright 2015, IEEE. (E) Schematic of integrated nanofluidic concentrator with microfluidic droplet generator [143], copyright 2011, American Chemical Society. (F) Schematic of continuous flow droplet-based concentrator showing the working principle of ICP [47].

Further, several studies have used ICP for the continuous concentration and extraction of diluted samples. This leads to the prevention of the dispersion of the concentration band where downstream processing is performed. Chen et al. developed a microfluidic device, where a droplet generator was incorporated with a biomolecule concentrator that uses ICP. The concentrated biomolecules became encapsulated in the droplets generated. The fabricated device used a flow-focusing configuration in the creation of droplets [143]. Later, Yu et al. proposed a pressure-assisted method to adjust

the position of the concentration band at the ejecting nozzle. The method enables maintenance of the obtained concentration. Phan et al. then proposed an approach to concentrating samples in continuously generated droplets. The tuning of the device was performed considering the flow rate and applied voltage [47, 144]. A recent study by Phan et al. reported on the use of ICP integrated with an aptamer probe for the continuous monitoring of target biomolecules in the serum secreted from a patient. The device enabled obtaining a cleaned serum at the outlet [46].

2.3 Paper Based Microfluidic Devices

The use of paper as a substrate material in analytical chemistry runs as far back as the 18th century, with litmus paper [145, 146], and this application has been followed by many researchers adding milestones to the field[147]. Paper microfluidics or microfluidic paper-based analytical devices (μ PAD), however, is a relatively new category of analytical platforms; it was introduced by Martinez et al. in 2007 [148] and indicates the rediscovery of paper as a substrate for analytical devices. Since then, there has been growing interest in this field. This interest is supported by various advantages offered by paper, such as (i) low material, fabrication, and operation costs [149, 150], (ii) simple and rapid fabrication process, (iii) no need for external forces in fluid handling, as passive capillary wetting through the porous structure supports the process, (iv) porous structure supporting effective immobilization of reagents through physical adsorption, (v) ease of storage and transport, (vi) disposable nature, (vii) compatibility with various analytical methods, such as colorimetric, electrochemical, fluorescent, or electrochemiluminescent [148, 151, 152], (viii) simple and rapid detection capability of devices [153, 154], and (ix)

ubiquitous availability in a wide variety of forms, from simple printing paper to highly engineered papers with specific features [55, 155]. Papers with specified porosities and thicknesses and with engineered substrates, such as paper and polymer blend or metal particulates/metal strings, offer biocompatibility and electrical conductivity to the paper. Further, the use of paper as an analytical platform provides the opportunity to chemically modify the paper to be compatible with the application [55, 156]. In addition to these properties offered by paper as a substrate, the μ PAD systems can display the properties of conventional microfluidic systems. Some of the properties are lower sample volume, reduced by several orders of magnitude, capability to manipulate samples in a convenient chip platform, control of flow characteristics with the laminar flow in channels, and rapid reactions when necessary for analysis [52, 55, 157, 158].

2.3.1 Paper as a Substrate

To date, most μ PADs are fabricated by using Whatman No. 1 chromatography paper as the substrate. This is composed of pure cellulose and has a relatively uniform thickness of 180 μm , and the structure consists of a porosity of approximately 0.69, permitting medium flow rates and considerably uniform wicking properties [53, 54, 151, 156, 159-161]. Still, subjected to the application, the type of paper used can vary in a vast range, from various types of chromatography papers [162] to commercially available print papers, paper tissues, and other paper types. Martinez et al. used paper towels, as they were an economical choice, and also ITW Technicloth paper, as it offers increased wicking speeds [163, 164]. Further, modifications to papers can be performed, either at the basic step of pulp preparation for the manufacturing of paper by adding the required

reagents, by grafting materials such as fluorinated monomers, other functional groups, or polymers to the paper [165-167], or even by saturating the paper with water, thus modifying the paper surface to be slightly anionic with water-swollen hydrogels of cellulose and hemicellulose [156]. For applications of paper as a supporting material in various microfluidic platforms or other devices, normal print paper or cardboard is used [168, 169]. Print paper is slightly hydrophobic, as it contains around 10% alkyl ketene dimer (AKD), a hydrophobic substance that binds chemically with cellulose [170]. Moreover, metal-coated paper has been used by several researchers for the fabrication of various electrical components. Mazzeo et al. used A238 and A550 paper consisting of an evaporated aluminium layer to fabricate capacitive touch pads [171], whereas Pal et al. used hydrophobic cellulose paper coated with a nickel layer to manufacture triboelectric generators, which were able to generate peak voltage signals of approximately 300V [172].

2.3.2 Types of μ PAD

μ PADs—which are also known as paper-based analytical devices (PAD), lab-on-paper (LOP), system on a paper, and so forth—are categorized depending on their device configuration or the sensing technique they use. Hence, μ PADs are often introduced as two-dimensional, three-dimensional, origami, microzone, or well plates. μ PADs that comprise one layer of paper can be identified as 2D systems. These can either comprise channels and reservoirs [148, 173, 174] or test zones, as in microzone plates [175-177]. Three-dimensional μ PADs are fabricated either by stacking patterned paper on top of one another, each separated by double-sided adhesive tape that is punched to provide connections between paper channels/layers [23, 164], or using origami, the famous art of

folding the papers to obtain the desired shapes [151, 178, 179]. The 3D devices made using the art of origami are also known as origami-based devices. Three-dimensional μ PADs are more advantageous than 2D μ PADs in several ways. Each layer of the devices can be composed of different papers accompanied by different properties, enabling them to perform numerous tasks in a single device, and they are important for networking large testing arrays by connecting channels or holes between layers [55, 164]. μ PADs are also categorized based on their activity as electrochemical or bioactive paper-based analytical devices/systems. Bioactive paper-based devices signify the relation between paper properties and bioactivity [156]. Papers are rendered bioactive through the immobilization of biosensors, which are often biolinks, enzymes, antibodies, phages, or DNA aptamers/primers [180-184]. The functionalization of paper with biomolecules is performed at different stages, such as during web formation or the finishing process or as a separate step of printing on manufactured paper [184]. Another type is microfluidic paper-based electrochemical devices, in which electrode systems are built onto the paper substrate for signal generation and analyte detection. These systems are abbreviated by many terms, such as μ PED, ePAD, and PED [172, 185-189].

2.3.3 Transport in Paper Networks

2.3.3.1 *Capillary Driven Flow*

The fluid flow in a μ PAD is essentially governed by the capillary flow, where flow propagates through capillary wicking through the porous structure of the paper. Hence, governed by the micrometre-range length scale of porous structures, the flow is often recognized as laminar, resulting in low Reynolds numbers [190-192]. For a paper

channel with a constant cross-section, the flow inside the channel follows the Darcy's equation for flow in a porous medium, with straight circular capillaries and constant fluid properties.

$$Q = -\frac{\kappa WH}{\eta L} \Delta P \quad (2.26)$$

Where Q represents the volumetric flow rate, κ is the permeability of the paper, WH is the cross-sectional area of the channel, η is the dynamic viscosity, and ΔP is the pressure drop over the length L along the direction of flow [190, 193]. The analogy of electric currents used in conventional microfluidic channels is also applicable to paper channels, and Darcy's law can be rearranged to visualize the same form as that of Ohm's law [192, 193]. Following this, Fu et al. demonstrated that a straightforward extension of this relation (Eq. 2.27) is able to describe the flow in N straight channels of different widths, connected in a series [193].

$$Q = -\frac{\Delta P}{\frac{\eta}{\kappa} \sum_{i=1}^N \frac{L_i}{W_i H_i}} \quad (2.27)$$

Here, ΔP stands for the pressure drop across the total length of the channel, and i represents the respective properties of the i^{th} segment of the channel with different widths. Further, for a paper device with multiple channels connecting to a common channel, Darcy's law states that the ratio of inlet channel length is equal to the sub-stream width inside the main channel [192]. Moreover, this one-dimensional propagation of the fluid

front in a porous medium can also be described by the Lucas–Washburn relationship (Eq. 2.28) for static viscous flows.

$$x(t) = \sqrt{\frac{\gamma r t \cos \theta}{2\eta}} \quad (2.28)$$

where $x(t)$ is the distance travelled by the fluid front, γ is the surface tension, r is the average pore radius of the porous media, t is time, θ is the contact angle, and η is the dynamic viscosity [190, 193-195]. Hence, this kind of flow takes the form of $x(t) \sim \sqrt{t}$. The relation is valid for lateral flow assays in which the length is no longer than the height of a vertical fluidic column when the negative gravity is equal to the positive capillary force. Further, the surface tension acts as a driving force for fluid flow through the porous structure, while the viscous resistance counteracts the flow determining the flow velocity [191]. This in turn results in reducing the velocities as the channels lengthen [190, 193]. Fu et al. studied the effect of the contraction and expansion of channel width on fluid front propagation and characterized the adaptability of the Washburn-like flow to explain one-dimensional flow in varying-width straight channels [193]. Later, Zhong et al. studied the effect of constant channel width on flow characteristics, demonstrating that all paper strips follow the Washburn-like flow despite having different channel widths [196]. In addition to these studies and characterizations, the effect of the substrate properties of paper on fluid flow is of paramount importance, as these in turn determine crucial parameters involved in theoretical explanations of flow. Xu et al. demonstrated the effect of fibre orientation on average pore radius and capillary distribution, highlighting that a higher degree of fibre orientation along longitudinal directions of channel results in a fastened

flow. Further, the significance of the source of fibre used in manufacturing paper, substrate-treating methods such as plasma etching, beating, and calendaring, and ultraviolet light treatment have also been studied to analyse their influence on substrate properties such as porosity, surface area, density, or wettability, which in turn regulate the flow velocities through porous paper structure [194, 195, 197].

2.3.3.2 *Electroosmosis*

Because paper is a porous media, the electroosmotic flow in paper is defined as it is in any other porous media. When paper is saturated with an electrolyte and placed in parallel in an external electric field E , the ions move in either direction, depending on their relative sign polarity, generating an electric current I to pass through the material. In addition, the electrolytes in the Debye layer experience a nonzero volumetric body force due to its net electric charge, hence the electrolyte flows in the Debye layer. This engenders viscous friction between the adjacent bulk solution and the Debye layer; hence, the bulk solution is dragged along with the migrating charge. The flow occurs in the absence of any macroscopic pressure gradient and exhibits the characteristics of a plug flow with an approximately planer velocity profile that differs slightly near the Debye layer. This phenomenon is defined as electroosmosis, and the electroosmotic flow velocity is given by U [198-201].

Conversely, under an applied macroscopic pressure gradient ∇P , the fluid moves through the paper with a Darcy seepage velocity U , and electrolyte movement in the Debye layer causes a perturbation in ion distribution and creates a macroscopic electric

current I in the absence of an external electric field. Hence, in the presence of E and ∇P , I and U exhibit the given relationships:

$$I = \sigma \cdot E - \alpha \cdot \nabla P \quad (2.29)$$

$$U = \beta \cdot E - \frac{K}{\mu} \cdot \nabla P \quad (2.30)$$

where σ and K represent electric conductivity and permittivity tensors and α and β represent coupling electroosmotic tensor coefficients.

Figure 2.8: Schematic illustrating that the electrokinetic effects in the electric double layer induce the fluid flow inside porous media. The velocity profile for the bulk electrolyte flow away from the EDL is approximately planer.

The plug flow inside the porous material can be expressed by the Smoluchowski equation for electroosmotic flow velocity (u_{eo}), where ϵ is the dielectric constant of the medium, ϵ_0 is the permittivity of the vacuum, E is the applied electric field parallel to the porous material, ζ is the zeta potential, and η is the dynamic viscosity of the fluid [200, 201].

$$u_{eo} = -\frac{\varepsilon\varepsilon_o\zeta E}{\eta} \quad (2.31)$$

Electroosmotic mobility (μ_{eo}) is expressed as follows:

$$\mu_{eo} = \frac{\varepsilon\varepsilon_o\zeta}{\eta} \quad (2.32)$$

2.3.3.3 Electrophoresis

Electrophoresis is the motion of a charged particle/ion relative to a fluid under the influence of an electric field surrounding it. Given that the fluid is fixed, the relative motion of the particles can be considered opposite to that of the electrolytes. As the particles are smaller and low Reynolds numbers are involved in the process, the Stokes model can be applied to the motion of these particles [61, 202].

$$qE = 6\pi r\eta u \quad (2.33)$$

Here, q is the effective charge of the particle, E is the electric field, r is the Stokes radius of the particle, η is the dynamic viscosity of the electrolyte, and u is the linear velocity of the particle. Electrophoretic mobility (μ_{ep}) of the ion is given as the linear velocity of the particle per unit of the electric field:

$$\mu_{ep} = u/E = q/6\pi r\eta \quad (2.34)$$

As mobility is defined by particle charge, radius, and solution viscosity, it is an intrinsic property of the particle for a given system.

Figure 2.9: Schematic illustrating electrophoresis of a positively charged particle under an applied electric field. Particles in contact with the solution gain a surface charge and attract counter-ions to form EDL. Counter-ions in the EDL move in opposite directions to the electrostatic force acting on the surface charge of the particles, leading to the movement of the liquid surrounding the particles. The motion is transferred to the retardation force acting on the particles as a result of solution viscosity. Thus, on a global scale, motion of charged particles is observed in a stationary fluid.

In addition, as the phenomenon is due to the presence of a charge interface (EDL) between the particle surface and the surrounding fluid, this mobility is dependent on the thickness of the EDL, which is unique to the system.

The performance of ICP on a μ PAD is a platform where a combination of these electrokinetic phenomena acts to gain a balance between them to provide the concentration of a given charged sample.

2.3.4 Fabrication of μ PAD

The basic concept is to pattern a hydrophilic channel on paper marked by hydrophobic barriers. This can be accomplished using various methods available for the patterning of paper. These are photolithography, use of an analogue plotter, ink jet printing/etching, cutting, wax/flexography/screen printing, plasma/laser treatment, use of

sticker templates, and spraying hydrophobic substances [55, 170, 203-207]. Among these, cutting does not follow the basic patterning technique. Instead, it uses a knife or electronic cutter to cut the paper, and it is then sealed between tapes to create the paper-based platform [120, 170].

Among the methods that follow the concept of patterning, various hydrophobic barrier substances are used, ranging from expensive photoresists such as SU-8, PDMS, relatively inexpensive wax, polystyrene, and extremely cheap alkyl ketene dimer to highly accessible scholar glue and waterproof eye pencils [55, 170, 203, 204, 208]. Depending on the binding of these substances to the paper, the patterning can be categorized under three principles as (i) physical blocking of pores by photoresists or PDMS, (ii) physical deposition of hydrophobic agents, such as wax, polystyrene, glue, or eye pencil material, and (iii) chemical modification of paper, using cellulose reactive agents such as AKD, which typically react with the OH groups of cellulose, imparting hydrophobicity to cellulose fibres.

Depending on the technique used, the patterning approach can be either single-step or multi-step. In single-step processes, the direct deposition of the hydrophobic agent on selected portions of the paper is achieved, and this is called selective hydrophobization. Another approach is entire hydrophobization, followed by selective dehydrophobization, where the entire paper is rendered hydrophobic and then etched to mark the hydrophilic channel using photolithography, ink jet etching, or plasma oxidation. The presence of hydrophobic agents, however, changes the wetting properties of paper. Further, upon deposition some methods require heating for the penetration of these substances through the paper.

Figure 2.10: (A) Schematic illustrating the steps for fabrication of a μ PAD using photolithography [148], copyright 2007, Jhon Wiley and Sons. (B) Schematic showing patterning of the paper channel using an eye pencil to define the design of the paper channel [208], copyright 2017, Elsevier. (C) Schematic illustrating the fabrication of a μ PAD by spraying scholar glue on paper, where the hydrophilic channel is defined by the magnetic mask [204], copyright 2017, Elsevier.

2.3.5 ICP on μ PAD for sample preconcentration

Several researchers around the world have studied this principle, and in almost all the designs, the perm selective membrane has been fabricated using different derivatives of Nafion, either membrane or solutions of different concentrations. Aside from the perm selective membrane material, studies have continuously focused on the substrate material, the paper channel thickness, the geometrical configuration of the device, the influence of external factors such as heat, the influence of sample characteristics such as volatility, and

the performance of the devices for the concentration of various samples. To summarize these studies, Gong et al. developed two classes of μ PADs to implement ICP and obtained a 40-fold concentration of fluorescent tracers in fully wet paper [44]. Later, they also demonstrated preconcentration of DNA on a μ PAD composed of Nitrocellulose paper by patterning Nafion for simultaneous preconcentration and separation of the Hepatitis B virus. They obtained a limit of detection of 150 copies/mL, using direct ICP for preconcentration [117]. R. J. Yang's group also studied the concept and fabricated a device where channel width is halved following the Nafion junction. They obtained a 20-fold concentration for this geometry of the device, whereas straight channel only provided a 10-fold concentration [115]. Further, they obtained a significant 944-fold concentration factor for Fluorescein in a double-sided wax-printed sallow paper channel using the ion enrichment zone as the sample plug zone. The concentration time was 30 minutes [56]. Hong et al. developed a μ PAD with a flow bifurcation system to implement ICP for the continuous concentration, obtaining a 33-fold concentration for fluorescent dye [209]. Hung et al. demonstrated the concept for multi-channel paper-based devices, demonstrating that as the number of channels increases, the concentration factor also increases [116]. Another study of ICP by Han et al. reported a 1000-fold concentration for FITC-BSA upon continuous supply of sample to the channel [41]. Liu et al. also demonstrated preconcentration of samples with a concentration factor of more than 100 in an enclosed paper device [118].

In contrast to this, Phan et al. introduced the use of off-shelf Nafion membranes to create the nanoporous junction in implementing ICP and achieved a 60-fold concentration factor within 200 s on a μ PAD [120]. Following this study, Gao et al. implemented ICP

on μ PADs for negatively and positively charged dyes by integrating commercially available CEM and AEM, respectively. The concentration factors observed were 10-fold for fluorescein sodium and FITC-BSA with CEM and 20-fold for Rhodamine 6G with AEM [57]. Han et al. recently studied ICP for blood-based serums on μ PAD and obtained an effective 5-fold concentration factor of human serum samples, using Nafion membranes as the nanoporous junction [58].

Figure 2.11: μ PADs developed for the implementation of ICP. (A)(a) Schematic illustration of a μ PAD with a straight channel with respective dimensions, (b) Fabricated device on an optical microscope stage [115], copyright 2015, AIP publishing. (B) Structure of a μ PAD fabricated with several layers of different material [120], copyright 2016, Elsevier. (C)(a) Schematic showing ICP interface with IEM on paper channel, (b) Schematic of the setup with fluorescent imaging system based on LED illumination and smartphone camera [57]. (D) (a) Schematic of ICP implementing μ PAD, (b) Fabricated device showing sample reservoir and buffer reservoirs using colour ink. Inset shows the SEM image of the side view of the channel with Nafion-embedded adhesive tape [58], copyright 2018, Elsevier.

The fabrication of most of the above-mentioned devices was performed using wax printing, and the incorporation of Nafion was performed through direct pipetting or surface patterning. The devices comprised various geometrical configurations and dimensions, and the conditions applied for the implementation of ICP also differed from device to device. Some of the devices used cutting as a method of fabrication.

However, it is evident that most of the studies were conducted for blood-based fluids or fluorescent dyes, indicating that the field of ICP is novel up to date and there is greater potential for this technique to be utilized in other fields, such as environmental monitoring.

2.4 Conclusion

This chapter provides a comprehensive review of the literature on transport in microfluidic/nanofluidic channels, ion concentration polarization and its applications, and paper-based microfluidic devices. Starting from basic electrokinetic theories, the phenomenon of ion concentration polarization is described conceptually and using governing equations. This is followed by a review of the literature on theoretical explanations for ion concentration polarization and its inherent qualities. The fabrication of the nanoporous junction is then reviewed, and the currently available methods and materials used are described.

The final section comprehensively reviews the paper-based microfluidic devices. Starting from transport in paper networks, it reviews the fabrication methods for paper-based microfluidic devices and the applications of these devices in the implementation of ion concentration polarization. The limitations of the fabrication methods are discussed in detail to provide a better understanding of the potential for these to be developed. Fabrication methods of microfluidic paper-based devices implementing ICP are still under development, such that there is ample room to develop better and more convenient fabrication processes for microfluidic paper-based device to implement ICP. Wax printing seems to be a viable option, but the process is time-consuming and requires bulk

equipment. In this scenario, it is necessary to develop novel and convenient fabrication methods for microfluidic paper-based devices that enable rapid and enhanced concentration of samples to extend the detection limits of the devices and render them suitable for onsite monitoring.

Considering the current applications of ion concentration polarization on microfluidic paper-based devices, the scope is limited to basic studies of fluorescent dyes and blood-based serums. Relying on the potential of ion concentration polarization, however, the challenges of extending the use of the concept to other fields, such as environmental monitoring, concern the complicated chemistry involved with the analytes, the lack of fluorescent-detecting probes suitable for the implementation of ion concentration polarization, and changing environmental conditions. It is important to maintain the perm selectivity of the membrane and the charged nature of the analytes during the process under different conditions, such as the ion concentration of the samples and changed pH. Further, the physical and chemical characteristics of the analytes—such as their molecular weight, charge, affinities towards nanoporous membranes, and diffusivities—should be considered when ion concentration polarization is extended to different domains of analytes, such as micropollutants.

Hence, for successful future applications, it would be highly desirable to characterize the phenomenon and develop a low-cost, easy-to-fabricate microfluidic paper-based platform enabling ion concentration polarization to be employed in different domains of analytes, such as pathogens, heavy metal ions, and antibiotic residues in water.

CHAPTER 3   ENHANCED   SAMPLE   PRE- CONCENTRATION   BY   ION   CONCENTRATION POLARIZATION   ON   A   PARAFFIN   COATED CONVERGING   MICROFLUIDIC   PAPER   BASED ANALYTICAL PLATFORM

As elaborated in literature review, microfluidic paper based analytical devices (μ PADs) represent a modest and feasible alternative for conventional analytical methods. However, the inadequate sensitivity of these devices limits the possible applications of μ PADs. In this scenario, inducing ion concentration polarization (ICP) on μ PADs has shown promising to overcome this limitation by pre-concentrating the analytes of interest. In this chapter, a μ PAD implementing ICP using an off shelf Nafion® membrane as the perm selective membrane is presented for achieving an enhanced sample concentration for the diluted sample solutions. Section 3.1 discusses the general background of the study while section 3.2 explains the working principle of designed μ PAD. Section 3.3 presents design concept for a novel, inexpensive and convenient fabrication of a μ PAD and experimental methods for the enhancement of sample concentration on a μ PAD. Section 3.4 comprehensively discuss the factors affecting ICP, their impact on concentration factor and concepts applied during the study to optimize the designed μ PAD. Finally, this section elaborates on the diluted sample preconcentration on the optimized μ PAD to significantly enhance the concentration factor.

3.1 Introduction

Use of microfluidic chip devices in sample analysis is taken to a new era with microfluidic paper based analytical devices (μ PADs) for analytical chemistry applications such as separation of small molecules like protein and amino acids [55, 148, 170, 203]. μ PADs are manufactured using paper as the substrate material. μ PADs pose numerous advantages, including low material, fabrication and operation costs, simple and rapid fabrication processes, fluid handling by passive capillary wetting through porous structure, porous structure supporting effective immobilization of reagents through physical adsorption, compatibility with various other analytical methods (namely colorimetric, electrochemical or electrochemiluminescence), easy to store and transport, disposability and above all the simple and rapid detection capability of devices [22, 23, 53, 151, 152, 170, 203, 210]. Paper can be found ubiquitously and available in a wide variety of forms from simple printing paper to highly engineered papers with specific features [170]. Among these, Whatman filter papers has been used in most applications while nitrocellulose, paper towels, ITW Technicloth and other paper types are also recognized as important paper substrates [55, 117, 151, 210]. Up to date, most of these developed μ PADs are designed barely for detection of analytes. And most of the devices proposed the use of colorimetric detection as a common qualitative and quantitative detection technique in which the μ PADs are coated or pre-deposited with detection arrays on specific portions of paper to serve as detection zones [23, 151, 170, 210]. Hence, in most situations, the limit of detection (LOD) depends on factors such as reactivity/ binding of analytes with the detection array, absorption characteristics, color decay etc. Some devices are reported for the use of electrochemical sensing and electrochemiluminescence

as the method of detection for inorganic, organic or biological sample analysis [152, 189, 211].

Concentration of analyte samples prior to analysis is a common practice in enhancing the output signals on microfluidic platforms. Several techniques, stacking methods like isotachopheresis (≈ 1000) and field amplified sample stacking, gradient focusing methods like isoelectric focusing and electric field gradient focusing, and other techniques such as electrophoretic and electrokinetic trapping are just to name some [42, 122, 212-214]. Most of these techniques inherit their own pros and cons. For the stacking methods, it requires longer channels for the concentration of larger volumes, and limitations in changing velocity limit the obtainable concentration factor; whereas during the field focusing methods each of them require specific conditions, for instance the isoelectric focusing can be applied to analytes that only have an isoelectric point. In the analyte trapping techniques, electrokinetic trapping is known to be a better performing technique which depends on ion concentration polarization than the other techniques in that category [42].

Ion Concentration Polarization (ICP) is a successful method which allows pre-concentration of charged samples. ICP, being an ionic transport phenomenon, occurs near an ion selective nanostructured membrane and is induced by the formation of an ion enrichment zone and an ion depletion zone, causing the ions in the solution to get accumulated at a narrow region at one side of the membrane. This is usually called the sample plug and can be used to pre-concentrate dilute samples [41, 56, 115, 120]. Many researchers studied on this principle, and in almost all the designs the perm selective

membrane was fabricated with different derivatives of Nafion; either off shelf membranes or solutions of different concentrations [41, 44, 46, 56, 115, 117-120, 205, 209, 215, 216]. Among the designed devices, both μ PADs and conventional microfluidic platforms were developed with substrate material like PDMS. Phan *et al.* developed a continuous flow droplet based concentrator which uses the phenomenon of ICP for the concentration of samples, enabling a 100 fold concentration of a sample with droplet sizes ranging from 20 – 25 μm [47]. During the studies with μ PADs, Yeh *et al.* obtained a maximum concentration factor (CF) of 944 folds within 30 mins which is believed to be the highest concentration factor reported [56]. Recently, Gao *et al.* and Han *et al.* reported obtaining a CF of 50 for Rhodamine 6G and a CF of 40 for human serum, respectively [57, 58]. However, despite the advantages possessed by μ PADs implementing ICP, the fabrication techniques are still under development and their performance is dependent on manual operation and manipulation of conditions applied during operation.

Here, we present a μ PAD that enables enhanced sample pre-concentration by using an off shelf Nafion membrane with a design of converging and encased sample transporting channels, thereby achieving a maximum concentration factor of 2019 folds for an aqueous solution of Fluorescein Sodium salt for the both side paraffin coated channel device. Also, the effects of evaporation and electroosmotic flow on sample pre-concentration are examined for one-side-coated and both-side-coated devices, showing the importance of channel encasement to attain a higher concentration factor while implementing ICP. Furthermore, the change of depletion layer length with applied electric field is studied. Additionally, we report a convenient, flexible and a less time-consuming fabrication process to manufacture μ PADs implementing ICP by direct cutting of paper,

paraffin, and off-shelf Nafion membranes into desired pattern and stacking and assembling them by oven heating with an applied pressure.

3.2 Working Principle

The phenomenon of ICP is implemented under an applied DC electric field such that ions (charged molecules) tend to move towards either cathode or anode. However, the presence of an ion selective membrane, Nafion across the path allows flow of only cations through the membrane while preventing movement of anions. This leads to a charge imbalance in the system. Hence, ICP tends to form an ion enrichment region in the cathodic side of the nano-structured Nafion membrane and an ion depletion region at the anodic side to regain the charge balance in the system. During the creation of the depletion zone, a repulsive force acts on the ions trapped at the anodic side, preventing the movement of both cations and anions through the Nafion membrane. Thus, all the ions get accumulated at a narrow region of the anodic side of the ion selective membrane, commonly known as sample plug. In a converging channel, this trapping of ions at the anodic side gets more congested, leading to a denser and localized sample plug. Fig. 3.1 shows a schematic illustration for the ICP phenomenon on a fabricated μ PAD.

During our experiments, under application of a DC electric field, where a positive voltage is provided to the sample inlet using the common electrode connected to the power supply and the cathodic connection is placed over the Nafion membrane, flow of sample gets restricted by the depletion force and therefore the sample tends to be concentrated in the anodic side at a location closer to the Nafion membrane. The location of this sample plug is adjustable by variation of DC electric field. Furthermore, the

phenomenon of ICP can be influenced by the ionic strength and conductance of the perm selective membrane [41, 102, 115, 120]. However, it is not constrained by the hydrophobicity or binding characteristics of the molecules/ions or compound of interest.

Figure 3.1: Schematic illustration of the working principle of implementing ion concentration polarization (ICP) on a fabricated microfluidic paper-based device (μ PAD), EOF – Electroosmotic flow, EP- Electrophoresis, FICP – Depletion force created by ICP, F_{ca} – Capillary Force which is due to certain amount of the liquid sample left at the inlet reservoir under applied electric field.

3.3 Experimental Methods

3.2.1 Device Fabrication and Concept

The device was fabricated with a straight channel configuration which comprises with a sample inlet channel and an absorption channel with two reservoirs at both ends of the channels. The channels are converged as they meet at a junction in the middle of the device where a Nafion membrane is located. Two types of devices as one-side-paraffin-coated and both-sides-paraffin-coated were fabricated. For simplifying the fabrication process, each of the designs was prepared using Silhouette Studio, custom software

designed for electronic craft cutting (Silhouette America Inc., Silhouette Cameo), and direct cutting was used as the fabrication technique. Whatman No.1 quantitative filter paper, paraffin films and Nafion membranes were all cut into desired size and shape with aid of the electronic cutter. In total, the μ PADs consist of four layers: the top and bottom paraffin layers, the Nafion membrane layer and the paper platform layer. An illustration of assembly of these layers is shown in 3-dimensional view in Fig. 3.2a. The overall dimensions of the device do not exceed $50\text{ mm} \times 20\text{ mm}$, indicating its suitability for on-site monitoring applications due to its compatible and smaller size. Fig. 3.2b illustrates the fabricated μ PAD. The device comprises with one in-built electrode which runs over the Nafion junction to supply negative voltage whereas a common electrode is used to supply positive voltage. Assembly of the components for the μ PAD was done manually on a glass slide following several steps of stacking of layers and heating in an oven at $80\text{ }^{\circ}\text{C}$ for a pre-determined time. The oven heating was performed in two stages: during the first stage, the bottom paraffin layer was adhered to the paper by heating for 5 minutes whereas the second stage was used to assemble the top paraffin layer to encase the paper channel with the Nafion membrane. Here, the heating time was varied to study its effect on channel thickness. During heating, paraffin films got melted and penetrated into the paper substrate, determining the thickness of the paper channel. This avoids the need of filter papers with different heights for fabricating paper channels with different thicknesses [55, 170].

Figure 3.2: (a) Schematic of a three-dimensional view of the designed microfluidic paper-based device, (b) The device with a straight channel geometry. (c) Effect of heating time on channel thickness. The bottom inset depicts the change of paraffin layer thickness with 2nd stage heating time; the top inset shows a comparison of the channel thicknesses for one side coated (2nd stage heating time - 0 min) and both side coated (2nd stage heating time - 3 min) devices

Fig. 3.2c illustrates the effect of heating time on channel thickness during device fabrication. The inset figure provides a figurative comparison of a device, fabricated with the first stage heating that comprises only of a bottom paraffin layer, with a device with the second stage heating and a top paraffin layer. The channel thickness got reduced with the second stage heating. Graphical illustration shows that, with increasing the second stage heating time, the channel thickness is reduced gradually as the higher the heating

time the deeper the penetration of paraffin into the paper substance. However, the reduction of channel thickness is affected by limited thickness of parafilm, leading to a slower reduction rate of the channel thickness as further increasing the second stage heating time. The increment in penetrated depth of wax into the paper is illustrated in the inset graph of Fig. 3.2c.

A detailed description for calculating the channel thickness is given in appendix A-1. Considering the observations made during device fabrication and analyzing the factors that affect channel thickness and experimental conditions, we set 3 minutes as the optimal heating time in the second heating cycle for the both sides paraffin coated device. To prevent the shrinkage of paraffin films and to maintain the alignment of paraffin film on top of filter paper during heating, a combination of paraffin film and filter paper was clamped in between two glass slides by using paper clips of similar size. These clips maintain the alignment between different layers of the device. Aluminum (Al) foil was placed between the glass slide and the paraffin film to avoid sticking of paraffin films to the glass slide. During the second stage of heating, Al foil was placed only between the top paraffin layer and the glass slide, causing the bottom paraffin layer to stick on one of the glass slides. Furthermore, use of a reusable anode electrode makes the fabrication process convenient and reduces the costs associated with fabrication and most importantly, the process results in fabrication of a disposable paper device in a convenient and a flexible manner within a short time. A detailed calculation of the fabrication cost and time can be found in appendix A-2 and A-3.

3.2.2 Material and Chemicals

Whatman grade 1 qualitative filter paper with its pore size 11 μm and thickness 180 μm (United Scientific – Singapore) was used as the platform of our μPAD in all the experiments. Nanoporous Nafion perfluorinated membrane sheets were purchased from Alfa Aesar (Thermo Fisher Scientific Inc.) laboratory to serve as the perm selective membrane, and paraffin film (Parafilm “M”) was used as hydrophobic substance to determine the channel thickness.

Fluorescein sodium salt ($\text{C}_{20}\text{H}_{10}\text{Na}_2\text{O}_5$) and Sodium chloride (NaCl , ACS reagent, $\geq 99.0\%$) were purchased from Sigma – Aldrich. Ultra-pure type-01 de-ionized water was obtained from a PURELAB Option – Q water purification system. All glassware was thoroughly washed with Iso Propyl Alcohol (IPA), rinsed with de-ionized water and oven dried prior to use. Fluorescein sample solution for pre-concentration demonstration was prepared by mixing 80 μM Fluorescein sodium solution with 100 mM Sodium chloride solution at 1:1 volume ratio. A series of Fluorescein sodium salt solutions with various concentrations of 500 mM, 100 mM, 1 mM, 100 μM , 1 μM , 500 nM and 100 nM were prepared for the calibration purpose. Fluorescein sodium salt was dissolved in ultra-pure de-ionized water for preparation of 500 mM fluorescein sodium solution. And a serial dilution was conducted from 500 mM to 100 nM solution using ultra-pure de-ionized water as the dilution agent. An ARE heating magnetic stirrer was used for preparation of solutions.

3.2.3 Experimental Setup

The experimental setup consists of a high voltage power supply (Stanford research systems, Inc., Model PS350/5000V- 25W), an inverted fluorescence microscope (Eclipse TE2000-E, Nikon) and a personal computer for data acquisition. A MIRO camera with Phantom Camera Control 2.14b was used in capturing fluorescence images acquired through the microscope, and an analysis of the images for several test parameters was done using ImageJ software. All the videos were recorded using a Canon 300D digital camera and analysis was performed using ImageJ and Microsoft Excel software.

3.4 Results and Discussion

3.4.1. Device Characterization

Prior to the demonstration of ICP for analyte pre-concentration, it is essential to characterize the device. Hence, the capillary flow through paper channel was studied as the initial step of characterization. Flow velocity through channels provides with information on self-driven flow of solution due to capillary action and essentially the effect of converging channel for the sample pre-concentration. Accordingly, to measure the flow of solutions through the channels of the one side paraffin coated and both side paraffin coated devices, 50 μ l of 100 mM fluorescein solution was placed on each device's sample inlet. Converging straight channel geometry was used as the paper based platform. The movement of solution through channel was captured in a video, and frames extracted from the video were used for calculation of travelled distance of sample with time by "ImageJ" software. Graphical representation of fluid front travelled distance vs time in both devices is shown in Fig. 3.3a and Fig. 3.3b.

Both illustrations show that, having a larger channel thickness, the one-side-paraffin-coated device yields a higher velocity than that of the both-side-paraffin-coated device. From the gradient of graphical representations, we can see that as the samples reach the converging region, there is a slight reduction in velocity; as the samples move out from the converging region, there is a slight increment in velocity. A graphical illustration for this converging - diverging section (10 mm to 15 mm) is provided in appendix A-4 to clearly show this effect as Fig. 3.3B illustrates the fluid front movement with time for the whole channel length. It indicates that the effect becomes more significant for the both side paraffin coated device. The variation of velocity in the converging region proves that there is a squeezing effect on the fluid flow, making it to concentrate at a more localized region and thus improving the concentration factor. Furthermore, the steep gradient at the beginning of the flow in each device indicates a pressure induced flow due to a pressure difference between the sample inlet and the absorption reservoir.

Also, we observed certain amount of sample liquid left in the inlet reservoir once the channel is fully wetted by capillary flow. This observation confirms that the sample volume of 50 μl is adequate and acts as an unlimited supply of liquid for the given channel length without drying off the channel during the process of implementing ICP. This is also further confirmed by the study on effect of evaporation in section 3.4.2.

Electroosmotic flow (EOF) of solutions within the paper channel is another important factor that affects the device performance. If the EOF velocity within the channel is reduced, a localized concentration plug with a higher fluorescence intensity can

be obtained [56]. Hence, the effect of paraffin coating on EOF was measured by using the current monitoring method [217, 218] at a fixed voltage of 400 V. For this, two PBS buffer solutions with different concentrations which slightly differ from each other were used. Current was measured referring to standard procedures and Fig. 3.3c shows the schematic of the basic procedure of EOF measurement for a microchannel together with the variation of current with time for devices with different channel thicknesses. The calculation of EOF velocity is provided in appendix A-5. It is seen that the one-side-paraffin-coated device yields a higher EOF which is 0.2 mm/s while the both-side-paraffin-coated device results in lower EOF. Moreover, EOF gets reduced as the channel thickness is reduced. For both side-coated devices, EOF velocities are 0.167 mm/s and 0.132 mm/s for the devices with second stage heating times of 3 minute and 5 minutes, respectively. This reduction of EOF is due to the restricted movement of solutions through limited thickness of the both-side-paraffin-coated devices as well as by lower zeta potential of paraffin wax, and it can be explained by Smoluchowski equation[61, 68, 202, 219-221] as

$$u = \left(\frac{\varepsilon \zeta}{4\pi\eta} \right) E \quad (3.1)$$

where ε is dielectric constant for the electrolyte, ζ is Zeta potential, η is viscosity and E is applied electric potential.

As the Zeta potential of system is reduced by both side coated wax, it leads to the lower EOF velocity of electrolyte.

Figure 3.3: (a) Images showing the flow through channel: scale bar = 10 mm. (b) Flow through channel in terms of distance vs time. Gradients representing the velocity in each region are: 0.39 mm/s before the converging region and 0.29 mm/s at the diverging region. The dashed lines represent the converging diverging region in the device. (c) Electropherograms showing current vs. time by using the current monitoring method for measurement of EOF. (d) Current vs voltage relationship on the μ PAD during ICP pre-concentration of samples; Region (I) – Ohmic region; Ohmic conductance = $4.67 \mu\Omega^{-1}$, Region (II) – Limiting region, Region (III) – Overlimiting region; overlimiting conductance = $2.12 \mu\Omega^{-1}$

Current vs voltage variation was studied as a measurable indication of implementing ICP. The measurements provide informative data to predict a working voltage range for a μ PAD to implement ICP. The general pattern of current vs voltage variation is shown in Fig. 3.3d. In region-I, initially the current increases linearly with

voltage following the Ohmic relationship and hence called the Ohmic region. Gradually, the current reaches an almost stable value with increasing voltage since the current is affected by the presence of the depletion zone, thereby forming region II. Hence, this region is named the limiting region and identified as the voltage range where the phenomenon of ICP can successfully be implemented. Further increment of the applied voltage results in increasing current flow with destruction of the depletion zone by electro-convection that occurs at relatively high voltages. Hence, region III is identified as the over limiting region [41, 56].

3.4.2. Effect of Evaporation of Solution on Sample Preconcentration

During sample pre-concentration on paper-based devices, the forward flow of solution in channels is governed by capillary flow. However, some of the solution gets evaporated into the surrounding from the open reservoir and open channels, where applicable, reducing the volume of sample available for pre-concentration. Hence, the droplet volume on the inlet reservoir in both, one-side-paraffin-coated and both-side-paraffin-coated devices were analysed experimentally.

The experimental setup consists of a solid stage for the device to reside, a long working distance lens attached to MIRO camera focusing on a predetermined spot on the stage to capture the droplet images, and an inverted Nikon 300D camera hanging over the device to capture the flow through channel. 50 μ l of 100 mM fluorescein sodium solution was inserted into the inlet reservoir of the device with a micropipette. It should be noted that, the inlet reservoir of the both-side-paraffin-coated device comprises of an open area to the atmosphere for introduction of samples. And, for the clarification of this

phenomenon, evaporation of a droplet having the same volume on a non-absorbing surface (glass) was also investigated. The analysis of the captured droplet images was performed with aid of ‘ImageJ- Low Band Asymmetric Drop Shape Analysis (LB-ADSA)’ tool. During the analysis, it was assumed that fluorescein sodium solution behaves similarly to water since a lower concentration aqueous solution of fluorescein sodium salt was used.

Figure 3.4: (a) Droplet volume on reservoir. The inset image shows a side view of the droplet on reservoir: scale bar = 1 mm. (b) Volume of sample absorbed into the porous channel for both devices. (c) Comparison of flow through channel with the combined effects of flow through channel and evaporation on droplet volume reduction. (d) Volume loss due to the sole effect of evaporation.

The results provide the combined effect of evaporation and flow through channel on volume reduction. Fig. 3.4a shows the droplet volume reduction on reservoir with time for different devices/ surfaces which are non-absorbing, one-side-paraffin-coated and both-side-paraffin-coated; while the inset figure shows the side view of a droplet captured by MIRO camera. This graph indicates that, the volume reduction of the droplet on non-absorbing surface follows a linear trend whereas for both the paper devices, the initial rate of the volume reduction is higher and then a gradual reduction is observed, which due to the combined effects of both evaporation and flow through channel towards volume reduction of sample droplet. This reduction follows a third order polynomial trend. The initial sudden volume reduction observed on paper-based devices is mostly supported by the dry and porous paper channel available for the flow as well as the pressure induced by the initial droplet volume on flow through channel rather than evaporation. As the time elapses, the volume reduction rate is reduced, since the channel gets saturated with continuous flow, nonexistence of pressure induced flow and reduced volume of the droplet remaining on reservoir. Furthermore, the droplet volume on the one-side-paraffin-coated device reservoir is greatly reduced than that on the both-side-paraffin-coated device reservoir, indicating the increased evaporation from open channel geometry and increased flow through channel due to larger channel thickness. Another interesting fact observed is that the volume reduction rate roughly doubles from the non-absorbing surface to the both side paraffin coated device and again from the both-side-paraffin-coated device to the one-side-paraffin-coated device. This confirms that the presence of paraffin coating exerts a positive effect on minimizing the evaporation of sample solution from the reservoir, and volume reduction is the combined effects of both evaporation and forward flow of

solution through channel. The times taken for 50 μl sample to completely dry on the non-absorbing, both-side-paraffin-coated and one-side-paraffin-coated surfaces are approximately around 142 min, 65 min and 24 min, respectively.

Since the analysis only provides the combined effects of evaporation and flow through channel, a separate analysis was carried out to identify the contribution from flow through channel towards volume reduction in the reservoir. For this purpose, the paper was considered as a porous medium and an area calculation was performed with aid of ImageJ. Hence, the following simple mathematical formula of calculating pore volume in a porous medium was adopted to calculate the volume of sample transported through channel,

$$V_p = \varphi \times V_b \quad (3.2)$$

where V_p , φ , and V_b stand for the pore volume, porosity, and bulk volume, respectively.

For a paper-based device V_b takes the form of

$$V_b = A \times t \quad (3.3)$$

and φ is defined as

$$\varphi = 1 - \frac{\rho_{paper}}{\rho_{fibre}} \quad (3.4)$$

where A is the area of the device covered by the solution, t is the channel thickness, ρ_{paper} is the bulk density of paper and ρ_{fibre} is the density of cellulose fibres used in filter paper.

For Whatman No. 1 papers, ρ_{paper} and ρ_{fibre} take the values of 483 kg/m³ and 1540 kg/m³, respectively [156].

It is assumed that the porous channel becomes saturated during flow and there are no void spaces within the area wetted by the solution. The paper porosity does not affect the volume calculations based on our observation that part of the solution is absorbed immediately by the paper in reservoir area once it is introduced to the sample inlet reservoir. In the calculations, the porosity of Whatman No. 1 filter paper - 0.69 was used. Fig. 3.4b shows the sample volume vs time for both devices. Clearly, the volume absorbed into the channel increases almost linearly with time for both devices. Also, it suggests the higher flow through the channel in the one-side-paraffin-coated device mainly due to its higher channel thickness. Fig. 3.4c shows a comparison of the combined effects of evaporation and flow and the effect of flow through channel on the volume reduction in reservoirs of the two paper-based devices. The volume reduction on the single side paraffin coated device is greatly contributed by evaporation, thereby reducing the available volume of sample for preconcentration. This is one of the most important reasons that preconcentration on the one-side-paraffin-coated device to end up at a lower concentration factor. Fig. 3.4d depicts the volume loss due to the sole effect of evaporation for the two devices. The figure provides the cumulative volume evaporated with time. Initially, there is a significantly higher-volume reduction within a certain time in the one-side-paraffin-coated device due to evaporation when compared to the both-side-coated-device. Thereafter, the volume reduction on the one side coated device is not observed, indicating that the volume reduction is governed by the flow through channel. However, for the both-side-paraffin-coated device, the initial volume reduction is

supported by flow through the channel rather than by evaporation. The comparison of volume loss rate also implies that the top paraffin layer acts as a barrier for the sample evaporation and it greatly inhibits the evaporation of solution from the paper channel. It provides with an adequate time for the device to draw the sample into the channel prior to evaporation.

3.4.3. Preconcentration of Sample by ICP

Fig. 3.5a shows the graphical representation of sample concentration in the one-side-coated device while Fig. 3.5b shows the same for the both-side-coated device. In the both-side-coated device, a maximum fluorescence intensity of 223.4 was attained within the pre-concentration duration of 803s, corresponding to a fluorescein concentration of 80.78 mM. This results in a CF of 2019 which is the highest reported up to date to the best of our knowledge. The initial concentration of fluorescein sodium salt in the solution used for the implementation of ICP was 40 μ M. A detailed calculation of concentration factor determination is provided in appendix A-6. As for the one-side-coated device, the observed CF was only 62.5, much lower than that of the both-side-coated device. The resulting higher concentration factor in the both-side-coated device can be attributed to the combined effects of lower EOF and much smaller sample evaporation during the process; these are resulted from both side paraffin coating and the effect of channel converging geometry in the pre-concentrating region.

At lower concentrations, within the paper channel of the one-side-paraffin-coated device, the fluorescein seems to saturate rapidly, giving rise to a higher intensity value for the same fluorescein concentration than that obtained for the both-side-paraffin-coated

device. This is supported by the higher channel thickness of the one-side-paraffin-coated device which allows the sample to move forward within the channel rapidly. However, the evaporation from the top surface of the one-side-paraffin-coated device lowers down the maximum attainable fluorescence intensity of the device; whereas the both-side-paraffin-coated device has an encased paper channel, thereby achieving the higher maximum fluorescence intensity.

Since the concentration at the anodic side leads to the concentration band movement further towards the anode with changing voltages, a scaling analysis was conducted to determine the depletion length increment with voltage. Fig 3.5c shows the depletion length scales as a quadratic power of applied voltage. This however does not support Dukhin's prediction[82] that the depletion layer thickness decreases inversely with electric field [76]. This scaling of the depletion length as a quadratic power of applied voltage provides a means of adjusting the system performance to obtain the concentration band of desired samples at a predetermined position on the anodic side of the channel through regulating the applied voltage within the limiting region.

The sample solutions of fluorescein sodium salt used during the experiment were mixed with NaCl. The addition of NaCl in 1:1 volume ratio to fluorescein sodium solution serves the purpose of increasing ionic concentration in solution and hence rapid implementation of ICP to preconcentrate samples. The phenomenon behind this is that as the concentration of ions increases near the Nafion membrane, the creation of ion depletion zone is observed sooner than that at a lower ion concentration case, leading to a fast implementation of ICP.

Figure 3.5: (a) Fluorescence intensity vs time for the one side coated straight channel geometry. The inset shows the calibration curve for the one side coated device. (b) Fluorescence intensity vs time for the both side coated straight channel geometry. The inset shows the calibration curve for the both side coated device. (c) Depletion layer length vs applied voltage. The depletion length scales as a quadratic power of applied voltage.

3.5 Conclusion

μ PADs present a modest and feasible way of sample analysis compared to the conventional methods. However, the devices' possible applications are limited by their

inadequate sensitivity. Further, the current methods of fabricating these μ PADs comprise of various disadvantages discussed in detail in chapter 2, section 2.3.4. Therefore, in this study, we presented a convenient, flexible and less time-consuming fabrication process for μ PADs using Whatman filter papers and laboratory paraffin films, thus reducing both fabrication time and cost significantly. The device fabricated following a featured design with encased converging channels at the middle has achieved significantly enhanced concentration factors of more than 2000 for the straight channel geometry. Our studies showed that this enhanced concentration factor is due to the suppressed EOF, reduced evaporation of sample as well as squeezing effect of the converging channel geometry. Furthermore, for the one side paraffin coated straight channel device, a concentration factor of 62.5 was obtained. The reduction of the concentration factor is greatly contributed by evaporation of sample through open channel geometry, hence less sample available for preconcentration. Also, we showed that the paraffin coating on either side of paper channel serves as a channel thickness determining agent, a good hydrophobic barrier substance that suppresses the EOF and an encasing for the paper device which minimizes the evaporation of sample. Additionally, we found that the depletion length in the μ PAD scales with a quadratic power of applied electric voltage. Nonetheless, the present simply fabricated, inexpensive device shows a potential for a broader range of analytical applications from laboratory sample preconcentration, point of care diagnostics to environmental monitoring.

The feasibility of a μ PAD fabricated following this method for the detection of pathogenic microorganisms via performance of ICP is comprehensively studied in next section.

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CHAPTER 4   RAPID   PRE-CONCENTRATION   OF *Escherichia coli* IN A MICROFLUIDIC PAPER-BASED DEVICE   USING   ION   CONCENTRATION POLARIZATION

As discussed in previous chapter, the phenomenon of ICP has the potential to be applied in the field of environmental monitoring. Here, the focus is centered on monitoring of aqueous micropollutants of a wide variety with distinctive properties to each type of micropollutants are chemically as well as physically. In this chapter, the attention is given to monitor the pathogenic microorganisms in water recognizing the threat induced by these on human/animal health and environment. The purpose is achieved through rapid detection of pathogens by concentrating them on a μ PAD using ICP. *Escherichia coli* (*E. coli*) is used as the model pathogenic bacteria here. Section 4.1 provides a general background for the study explaining the risk of the presence of pathogens in water. Section 4.2 describes the working principle of the device for the selected bacterial strain on the device whereas section 4.3 illustrates the experimental methods followed during the study. Section 4.4 comprehensively elaborates the results of the conducted experiments under effect of various factors on ICP of selected type of sample and performance of ICP to achieve a rapid concentration of bacteria enabling rapid detection of them on a μ PAD.

4.1 Introduction

Securing water quality is performed through continuous monitoring of different quality chapters such as physical, chemical or biological quality [7]. Among these, the

microbial quality of water is assessed by examining the level of fecal indication bacteria; i.e., *Escherichia coli* (*E. coli*) and enterococci, presence in water [222]. *E. coli* has been identified as a strongly related bacterium for the presence of pathogenic fecal forms in water and hence the USEPA recommends examining levels of *E. coli* to ensure the microbial quality of water [10]. *E. coli* is a gram-negative bacterium present in variety of strains and can be categorized under normal intestinal microbial flora. Though many strains of *E. coli* are not pathogenic, some strains are responsible for causing either intestinal or extraintestinal diseases. Diarrhea, urinary tract infections and meningitis are some examples for these diseases [24, 222]. The strains isolated from environmental waters are often found to be pathogens carrying either toxin genes or other virulence genes [222, 223]. These strains of *E. coli* usually end up in environmental waters either following the discharge of effluent from Sewage Treatment Plants or from animal excrement. Some reported cases of water borne *E. coli* outbreaks are closing more than 32% of US beaches monitored by USEPA in 2005-2006 due to fecal contamination, water borne disease outbreak caused by untreated private water supply contaminated by animal excrement in Scotland in 1999, and 1991 water borne infection outbreak among bathers due to swimming in the fecally contaminated lake water in lakeside park near Portland [10, 11, 224].

However, the conventionally available methods of *E. coli* detection are mostly dependent on cell culturing or other molecule-based cell amplification techniques. Culturing of cells on a nutrient rich medium multiply the cell population to a detectable level starting from a lower cell concentration. These methods are time consuming (24 – 48 hours of cell culturing) and labour intensive, making them impractical for rapid detection;

whereas for *E. coli* the concentrations vary greatly during a day's time depending on the factors like amount of sunlight, location, time of the day, wind speed and *etc.* [10, 24-27]. On the other hand, cell amplification techniques such as polymerase chain reaction (PCR) or isothermal amplification provide high selectivity and sensitivity as they are based on DNA amplification of targeted bacteria. However, these amplification techniques possess limitations such as precise environmental condition maintenance, complicated sample preparation, use of specific equipment and requirement for skilled laboratory personnel. Also, these methods require handling large quantities of media and other solutions during the process of cell enumeration which end as biological waste [24, 27, 28]. Under such circumstances, microfluidic devices become a viable solution by offering advantages like reduced sample quantities, reduced assay time and portability [216, 225, 226]. Up to date, several microfluidic devices have been proposed for the detection of *E. coli* based on cell capturing with aid of various biological receptors or cell amplification. [24-28]. Li *et al.* developed a quartz crystal Au electrode based electrochemical immunosensor with a LOD of 10^3 CFU/mL and a detection time less than 10 minutes [227]. Safavieh *et al.* developed a electrochemical biosensor that enables loop-mediated isothermal amplification with a detection capability of 48 CFU/mL in 35 minutes [24]. However, these conventional PDMS microfluidic devices were fabricated following complex and time consuming processes such as soft lithography, micro milling, *etc.* and materials used are comparatively expensive [24, 52, 157, 228]. Also, these devices need to be either biologically active to capture the cells or require the use of complex sample preparation steps during the operation [24-26].

To solve these issues, microfluidic paper based analytical devices (μ PAD) to concentrate *E. coli* to a detectable level can be used as a viable option. In addition to the advantages offered by conventional microfluidic devices, μ PADs have numerous advantages like their lower material, fabrication costs, passive capillary wicking enabled sample flow, compatibility with various sensing methods (e.g. colorimetric, fluorescence, electrochemical, *etc.*,) and simple and rapid fabrication processes [55, 151, 152, 203, 229]. In order to concentrate *E. coli* there are microfluidic techniques such as ICP, Field amplified sample stacking (FASS), isotachopheresis (ITP) or bi-phase induced charge electroosmosis (BICEO) [41, 56, 230]. ICP, an electrokinetic phenomenon that enables amplification or concentration of charged particles/ ions into several orders of magnitude, has been studied by several researchers for its characteristics such as non-linear electrokinetic flow, over limiting current, ion-depletion zone, micro vortices under different conditions such as electrothermal convection, natural convection, chemical reaction and charge regulation [69, 76, 77, 80, 231]. The phenomenon has employed in concentrating analytes, proteins, viruses and other fluorescent dyes on μ PADs by multiple researchers [41, 44, 56, 115, 117, 120]. Yeh *et al.* observed a concentration factor of 944 folds for the fluorescein and 835 folds for fluorescein isothiocyanate conjugated bovine serum albumin (FITC-BSA) [56]. So far, the technique is not used for the pre-concentration of *E. coli* which is a gram-negative bacterium.

In this study, we focus on implementing ICP to pre-concentrate *E. coli* using *E. coli* ATCC 25922, a laboratory strain of *E. coli*, on a μ PAD to achieve a higher concentration factor in a shorter time. The μ PAD with a simple straight channel geometry consisting of reservoirs at either end is fabricated following a simple and rapid fabrication

process of direct cutting and oven heating; this method is inexpensive, disposable and easy to use.

4.2 Working Principle

ICP is an ionic transport phenomenon occurs near perm selective membrane (*i.e.*, Nafion®) under the presence of a DC electric field by the formation of an ion depletion zone and an ion enrichment zone [46, 47, 72, 106, 119, 232]. Under the DC electric field, ions tend to move towards either cathode or anode. However, since Nafion® restricts the movement of anions through the membrane, there occurs an increment of ions at cathodic side of the membrane. This leads to an ion imbalance in the system, leading to an ion enrichment zone created on cathodic side of the membrane and a depletion zone created at the anodic side of the membrane. Hence, at the edge of the depletion zone, both cations and anions get concentrated thereby forming a concentration band. This enables the ICP as a pre-concentration technique for dilute sample solutions [41, 115, 120]. The resulting concentration factor from ICP is dependent on factors such as electroosmotic flow (EOF) through channel and sample solution used [56] Fig. 4.1A shows a schematic of the ICP phenomenon.

Being a gram-negative bacterium, *E. coli* consists of a thin cell wall covered by phospholipids and lipopolysaccharides. The lipopolysaccharides induce a strong negative charge on *E. coli*, enabling it to behave as negatively charged particles [233, 234]. This allows us to implement ICP for pre-concentration of *E. coli* suspended in an aqueous solution. While the EOF of bulk solution is from anode to cathode, an electrophoretic force is acting on cells in the opposite direction due to negatively charged nature of *E. coli*.

During implementation of ICP at the edge of depletion zone, the concentration of *E. coli* occurs forming a localized concentration band at the anodic side of the nafion membrane due to the equilibrium of forces acting on *E. coli* cells. The working principle for implementing ICP is illustrated in Fig. 4.1B.

Figure 4.1: (A) Schematic illustration of ion concentration polarization (ICP) phenomenon at a perm selective membrane. (B) An Illustration of the working principle of ICP for pre-concentration of negatively charge *E. coli* cells on a microfluidic paper based device

4.3 Experimental Methods

4.3.1 Device Fabrication

The device fabricated comprises of four major layers each which are designed using custom software Silhouette Studio and cut into the shape using the electronic craft cutter (Silhouette America, Inc., Silhouette Cameo) [120]. From top to bottom the layers of the device are a top paraffin layer, an off-shelf Nafion® membrane, a Whatman No. 1 chromatography paper layer and a bottom paraffin layer. Two bottom layers are assembled by heating in the oven at 80°C for 5 min prior to cutting. Then, the Nafion® membrane (2 mm × 5 mm) is placed at desired distance from the inlet reservoir; in this case 15 mm, and the microwire electrodes (ϕ 100 μ m) are assembled into the device.

Finally, the top paraffin layer designed to encase the paper channel is super positioned on the paper channel and the assembly is clamped between two glass slides secured with Al foils for final oven heating at 80°C for 3 min. Fig. 4.2A illustrates the schematic of the device. The fabricated paper channel is of 2 mm × 30 mm (width × length) with a height of 152 µm once fully fabricated. A schematic of the device with dimensions is included in appendix B-1. For the device fabrication, Whatman No. 1 filter paper is from United scientific (Singapore), off-shelf Nafion® 212 membranes are from Fuel Cell Store (Texas), and laboratory paraffin film (Parafilm M) is used as hydrophobic barrier substance.

4.3.2 Sample Preparation

Bacterial cells of *E. coli* ATCC 25922 are grown in Luria-Bertani (LB) medium overnight following the inoculation of medium with previously maintained cultures. Upon the growth of *E. coli*, diluted sample solutions of 1×10^5 CFU/mL are prepared in NaCl of varying concentrations as 0.2 M, 0.4 M and 0.6 M. The samples are stained with 20 µM SYTO 9 green fluorescent nucleic acid in 1: 1 volume fractions prior to final use for pre-concentration tests. This dilutes the concentration of bacterial cells and NaCl into half of the initial concentrations; i.e., 5×10^4 CFU/mL for bacterial cells, and 0.1 M, 0.2 M and 0.3 M for NaCl, in prepared test solutions. Sodium chloride (NaCl, ACS reagent, ≥ 99.0%) is from Sigma – Aldrich, SYTO 9 is from ThermoFisher scientific (Singapore). *E. coli* ATCC 25922 is from Bio-Rev Pte Ltd (Singapore), and LB nutrition medium is from Becton & Dickinson Company (France). All the reagents are prepared using ultrapure type 1 water obtained from Direct – Q 5UV water purification system.

4.3.3 Experimental Setup

Fig. 4.2B illustrates the experimental setup that consists of a power supply (Stanford Research Systems, Inc.; Model PS350), an inverted microscope (Nikon Eclipse TE2000 – U), a laser source to excite the cells stained with SYTO 9 (excitation wavelength 473 nm), a FITC filter with Ex 465 – 495 nm, DM 505 and BA 515 – 555 (Nikon Eclipse E800 series) as the fluorescence filter, a CMOS camera (Phantom Miro4–M VR1208, Resolution 800×600 , frame rate 20 s⁻¹, exposure time 30,000 μ s, bit depth 8) for capturing the images and a computational interface for data acquisition. This customized setup allows for EOF measurements and calibration of fluorescence intensity. The “ImageJ” software is used for analysis of the acquired images. The fluorescence spectrum for SYTO 9 is included in appendix B-2.

Figure 4.2: (A) Schematic of the microfluidic paper-based device. (B) A schematic of the experimental setup used for concentration of *E. coli* by ICP experiments.

4.4 Results and Discussion

4.4.1 Characterization of μ PAD

Implementation of the ICP on a μ PAD requires the maintenance of certain operational conditions like applied voltage which depends on factors such as sample solution of interest, device configuration, *etc.* [44, 56, 117]. Hence, the device has been characterized to identify the optimum operational conditions.

4.4.1.1 *Electroosmotic Flow Through the Paper Channel*

EOF through the paper channel greatly influences the concentration factor observed during ICP. Suppressing the EOF of solution through channel enables ions/charged particles to stack into a dense localized concentration band with higher fluorescence intensity [56]. Hence, during this study, the effect a cell suspension on EOF suppressing is studied. The measurements are taken using the current monitoring method at a constant voltage (450 V); the standard EOF measuring procedure for microfluidic devices [217, 218]. Two sets of experiments are conducted with two different concentrations of NaCl solutions (8.5 mM and 10 mM). For the first set, pure NaCl solutions are used whereas for the second set, *E. coli* cells are suspended in both NaCl solutions. The *E. coli* concentration in each NaCl solution is 5×10^4 CFU/mL. Fig. 4.3A shows the current through channel with time for both the *E. coli* cell suspended NaCl and the pure NaCl solution used to study the EOF behaviour. It is seen that compared to the case of the pure NaCl solution, there is a reduction in EOF velocity and the steady state current for the *E. coli* cell suspension. Once the EOF is measured referring to the standard

procedure, a lower EOF velocity of 0.118 mm/s is reported for *E. coli* cell suspended NaCl while for pure NaCl solution it is 0.18 mm/s.

The reduction observed in EOF velocity for *E. coli* cell suspended NaCl is caused by the resistance induced to the flow when transporting bacteria through a porous medium. Being bacteria, *E. coli* shows a tendency to retain attached to the porous of the paper leading to partial clogging of the pores in paper while they are transported through the porous paper channel [235]. Further, this retention or clogging of porous structure can possibly be supported by the geometry of *E. coli* and pore structure. This is because the collection of flagella at the rear of *E. coli* cells is typically 7 -10 μm long and shows rotating movements. This may lead to clog the pores of paper with a typical diameter of 11 μm as *E. coli* behaves as swimmer bacteria due to flagella movements [236]. However, under an applied electric field, the electrokinetic movement of *E.coli* is in the direction of electric field and hence this clogging can be reversible by the action of electroosmotic drag [237]. Secondly, the reduction in steady state current for *E. coli* cell suspended NaCl can be attributed to the partial replacement of NaCl by *E. coli* cells during the flow and hence it reduces the conductivity of this cell suspension. The cell suspensions are proven to have a higher electrical resistance than that of their suspending liquid as their cell membrane is only partially permeable to the salts and hence induces a resistance to the flow of ions reducing the steady state current [238]. Further, the previously explained clogging of the porous structure by *E. coli* cells may lead to an increment of the electrical resistance of wetted channel due to the decrease of cross sectional area of conductor. [72, 106]. Also, the paraffin encasement of the device also suppresses the EOF to some extent due to the lower Zeta potential of paraffin wax compared to a paper channel without wax

encasement [56, 239]. However, here this effect remains similar as same device geometry is used for both NaCl solution and *E. coli* cell suspended NaCl. The combined effect of all the aforementioned factors reduces the EOF velocity and enhances the stacking of cells in a localized area resulting in a significantly high concentration factor.

4.4.1.2 Working Electric Potential Range

ICP is implemented under a DC electric field provided that a depletion zone is created. Hence, current vs applied electric voltage variation is analyzed to identify the working potential range. Further, as *E. coli* can be found in a wide variety of environmental waters of different ion concentrations, the experiments are carried out for different ionic conditions. Three sample solutions of NaCl with background concentrations of 0.1 M, 0.2 M and 0.3 M with a concentration of *E. coli* 5×10^4 CFU/mL in each solution are prepared as explained in Section 4.3.2. Fig. 4.3B illustrates the current vs applied electric voltage variation for different ion concentrations. For all three graphs, it is observable that initially the current increases linearly with the electric voltage following the Ohm's law and this region is known as the Ohmic region. Then the current remains constant for some range of electric potential which is known as the limiting region and the current in this region is called the limiting current. This current limitation is due to the generation of the depletion zone and hence lack of charge carriers in the channel occur by diffusion limitation of ions through Nafion® membrane. The depletion zone expands as the voltage is increased [76, 80, 240]. Hence, concentration of charged analytes occurs at the edge of the depletion zone during this electric potential range, forming a localized concentrated band of diluted sample solutions. However, further

increment of electric voltage leads to destruction of the depletion zone, thereby increasing the current flow through channel. This region is known as the over limiting region [56]. This over limiting current is associated to the water splitting which introduces H^+ and OH^- to the system and the strong convective mixing related to the second type of EOF at the face of Nafion® membrane at sufficiently high voltages. Such phenomenon destroys the concentration polarization within the system leaving only limiting region to be used during ICP [72, 76, 79, 231, 240]. Further, the graphs clearly show that with the increment of total ionic concentration of the solution the electric voltage corresponding to the beginning of limiting region decreases. Also, the current flow through channel gets increased with the increment of ionic concentration. This is because, with the increment of ionic concentration of the solution, the number of charge carriers flow through the channel increases and hence the current flow through channel increases. This leads to the generation of depletion zone at a lower electric voltage. Also, it is observable that current reaches over limiting region at a smaller voltage as the background salt concentration is increased. As the salt concentration gets increased, system must contain a higher ionic flux at the anodic region adjacent to the depletion zone. This makes convective mixing within the system faster and vortices formed to be larger leading to the destruction of depletion zone at a lower voltage [240].

This indicates the ability to overcome requirement of a higher applied electric voltage; one of the major bottlenecks of ICP for point of care (POC) diagnosis by increasing the total ion concentration of the solution. It is important that the total ion concentration is increased in such a way that it does not affect the signal intensity of the targeted analyte [241]. And it is observable that the current tends to reach saturation with

the increment of ionic concentration of NaCl. Such effect of saturation current can be attributed to the transportation of ions through a porous medium where the maximum ion transport is determined by pore saturation; i.e., at a low electrolyte concentration the pores are partially occupied by the ions and as the electrolyte concentration increases, eventually the pores get fully occupied and saturated limiting the number of ions transport through the porous media. This leads to the saturation of current through the porous paper channel as NaCl concentration is increased mainly due to the membrane saturation [69, 242, 243].

Figure 4.3: (A) Electropherograms showing current through channel vs. time for the comparison of electroosmotic flow (EOF) velocity through the paper channel for a NaCl blank solution and an *E. coli* cell suspension.; (B) Current vs. voltage relationship for *E. coli* cell suspensions in NaCl solutions with three different concentrations which are 0.1 M, 0.2 M, 0.3 M. Shown in dotted lines are (I) Ohmic region, (II) Limiting region, and (III) Over limiting region of voltages.

Further, as described in section 4.4.1.1 for the electric conductivity of cell suspensions becomes lower than that for the liquid solution. Hence, during the ICP process the limiting current for a cell suspension would have reached at a higher voltage than that for an ionic solution. This implies that working electric potential range depends

on the analytes of interest under the experiment and indicates the possibility of further reduction of applied electric potential during ICP for solution type samples with a higher ion concentration.

4.4.2 Preconcentration of *E. coli* Using ICP

The use of ICP for concentration of stained *E. coli* suspensions in different NaCl solutions is studied under various electric voltage. The applied electric voltages for background NaCl concentrations of 0.1 M, 0.2 M and 0.3 M in suspension are 90 V, 70 V and 50 V respectively. All the chosen voltages fall within limiting region where ICP can be implemented to preconcentrate analytes and are chosen from the current vs voltage curves obtained for respective background electrolyte concentrations. During the experiments, it is observed that for lower NaCl concentrations, even with a higher applied electric voltage the fluorescence intensity observed during ICP is significantly low and the enhancement of the fluorescence intensity is rather slow. The concentration band obtained also is narrower. However, for the *E. coli* suspended in 0.3 M NaCl solution, significantly high fluorescence intensity is observed with the progression of ICP and it reaches a maximum within 20 min saturating the paper channel. A considerably wide and intense concentration band is created for this cell suspension sample. Fig 4.4 shows the fluorescence intensity vs time for *E. coli* suspended in different NaCl concentrations with both figurative and graphical illustrations.

Since, the highest fluorescence intensity is observed for the *E. coli* cells suspended in 0.3 NaCl, the calibration of fluorescence intensity vs. cell concentration is performed for the stained cell cultures that are suspended in 0.3 M NaCl. A series of cell suspensions

ranging from 2×10^{10} CFU/mL to 2×10^3 CFU/mL is prepared in 0.6 M NaCl decreasing the concentration in 10 folds at each step. Then they are stained with 20 μ M SYTO 9 at 1:1 volume ratio adjusting the background concentration of NaCl to be 0.3 M in each cell suspension.

Figure 4.4: Implementation of ICP for the pre-concentration of *E. coli* with SYTO 9 as a fluorescent indicator; Fluorescence intensity vs. time for *E. coli* cell suspensions in different concentrations of NaCl solutions.; (A) Experimental images showing the concentration of *E. coli* suspended in different concentrations of NaCl on the anodic side of the paper channel (All captures are of same scale); (B) Quantitative results for the concentration of *E. coli* cells vs. time, suspended in different concentrations of NaCl.

The fluorescence intensity vs. concentration for varying cell densities in 0.3M NaCl is shown in Figure 5A. The results are comparable with the fluorescence intensities obtained by Xue's group [244]. Figure 5B shows the fluorescence intensity vs. time for cell suspension in 0.3 M NaCl with the concentration factor obtained. During this study, we can achieve a concentration factor of 2×10^5 folds according to the fluorescence calibration curve within minutes; this is considerably high and rapid. Baaske et. al also

reported a high concentration of nucleotides in hydrothermal pores by using the thermal convection and thermophoresis method [245].

During calibration of fluorescence intensity vs. *E. coli* concentration, it is observed that cell movement through the channel gets restricted due to the porous structure of the paper. Reasoning for this flow restriction is given in Section 4.1.1. Therefore, even though the calibration curve shows a higher intensity value for lower concentrations of cells, the initially observed fluorescence intensity during ICP remains low. In this scenario it is clear that NaCl solution first wets the channel. Afterwards, under the influence of electric field, the cells move through the channel and get concentrated near the depletion zone. Further, even though a high fluorescence intensity around 160 AU is observed during ICP, due to the limitations of calibrating fluorescence intensity at higher cell densities the exact maximum concentration factor corresponding to this high fluorescence intensity cannot be obtained.

A similar phenomenon may affect the lower fluorescence intensities obtained for *E. coli* suspensions in 0.1 M and 0.2 M NaCl. Since the flow of cells is initially suppressed and there are not enough charge carriers to induce sufficiently high force to move the cells through the porous channel, only a limited number of cells reach to the concentration band. However, it is seen that with time the fluorescence intensity increases slowly for these two samples as well. This indicates that NaCl solution concentration affects the speed of pre-concentration during ICP. With the increment of NaCl concentration, the amount of co-ions (i.e., Cl^- in this scenario) within the system also increases. And it is the amount of co-ions that repelled by the Nafion membrane defines

the depletion of ions/ charged particles in the system. Hence, as we increase the concentration of NaCl where *E. coli* is suspended, pre-concentration of *E. coli* happens faster [73].

Figure 4.5: (A) Calibration curve for *E. coli* cells stained with SYTO9 in terms of fluorescence intensity vs. *E. coli* concentration; background concentration of NaCl is 0.3 M; (B) Fluorescence intensity vs. time for *E. coli* cell suspension in 0.3 M NaCl indicating the maximum fluorescence intensity, readable maximum fluorescence intensities and corresponding cell concentrations obtained.

4.5 Conclusion

Pathogenic microorganisms in water poses a threat to the human/animal life and environment causing bacterial water borne diseases, reducing water quality and thereby making water bodies unfit for aquatic life and human recreational use. Concentrations of these pathogens in water vary drastically over short periods of time making the currently available time consuming methods inadequate to serve the purpose of rapid detection of aquatic microorganisms. Hence, during this study, the use of μ PAD implementing ICP for rapid pre-concentration of *E. coli* has been demonstrated. The proposed device is

inexpensive, and device fabrication follows a simple process. The device can achieve a considerably high concentration factor of 2×10^5 folds for *E. coli* within minutes. The suppression of EOF velocity by flow of *E. coli* cells through the porous paper channel lead the cells to concentrate at a localized area. The ICP can be implemented even at a lower voltage by increasing the total ion concentration of the sample. However, there is an upper limit to this increment as the electrical current gets saturated for the transport of ions through a porous medium. Hence, this can be proposed as a rapid pre-concentration technique over conventional time consuming methods of detecting *E. coli* during ballast water quality monitoring, coastal water quality monitoring for public beaches (to prevent outbreaks and minimize the damage) as sea water salinity is almost similar to the NaCl concentration used prior to staining of cells [10, 246, 247]. Further, with a simple sample preparation step of adjusting the total ion concentration of sample such as employing the relationship between conductivity measurement of sample and salt concentration [230], this technology can be applied for the pre-concentration of *E. coli* in fresh water as well.

In next section, the application of ICP will be broaden into the detection of aqueous heavy metal ions; a well-known micropollutant to cause adverse effect on human and animal health, where it studies the phenomenon comprehensively for heavy metal ions.

CHAPTER 5 DETECTION OF HEAVY METAL IONS IN AN AQUEOUS PHASE ON A MICROFLUIDIC PAPER BASED DEVICE USING ION CONCENTRATION POLARIZATION

Contamination of water bodies by heavy metals is of growing concern due to its adverse health effects; however, the presence of these contaminants in water bodies is in tracer amounts. This leads to the need for specific methods for the detection of these contaminants. The conventional methods available are expensive and time-consuming and require trained personnel. Therefore, during this study, I focused on the application of ICP for the detection of heavy metal ions, such as Ni^{2+} and Cu^{2+} , by preconcentrating them on an inexpensive μPAD .

5.1 Heavy Metal Ions

Heavy metals, also known as trace metals, are defined as “metallic elements with high atomic weights (e.g., mercury, chromium, cadmium, arsenic, and lead) that can damage living things at low concentrations and tend to accumulate in the food chain” by the United States Environmental Protection Agency (USEPA) [248-250]. In general, the atomic weights of these metals may range from around 63.5 to 200.6 g mol^{-1} , and their specific gravity is greater than 5 g cm^{-3} [8]. The USEPA lists 14 elements as priority heavy metals, according to their environmental impact. They are silver (Ag), arsenic (As), beryllium (Be), cadmium (Cd), chromium (Cr), copper (Cu), nickel (Ni), mercury (Hg), lead (Pb), selenium (Se), thallium (Tl), antimony (Sb), and zinc (Zn). Other than these,

noble metals such as gold (Au) and platinum (Pt), which are known as precious metals, can also be categorized under the label “heavy metals” [251].

5.1.1 Occurrence of Heavy Metal Ions in Nature

Heavy metals occur naturally on Earth’s crust and make constituents of rocks. Their existence in rocks is in the form of ores of sulphides or oxides [249, 252]. Subsequently, once these metals are extracted from these ores, it creates numerous ways of releasing these metals and their ions into the environment from the mines themselves, natural causes, and the numerous industrial processes that use these metals. The release may be either in elemental form or in ionic form [252]. Ultimately, they end up in water, increasing the concentrations of heavy metals in water [249]. Once the concentrations exceed certain tolerance values or permissible levels, water is said to be contaminated and unfit for use by humans and animals [8, 18, 252]. These toxicity levels or tolerance levels are heavily dependent on soil pH and speciation of metals in the environment. The metals capable of forming strong compounds become less toxic, whereas the metals that exist as ions exhibit higher toxicity due to their enhanced bioavailability [22, 248].

5.1.2 Heavy Metal Ions as Micropollutants

The presence of these heavy metals in water usually takes the form of heavy metal ions. Most of these ions are toxic at tracer concentrations and non-biodegradable and also lead to bioaccumulation in plant and animal bodies along the food chain. [39]. Further, metal ions are the most toxic at their most stable oxidation state (e.g., Cu^{2+} , Ni^{2+} , and Ag^{+}) [248, 253], and even though some of the heavy metal ions are known as micronutrients (e.g., Cu, Zn, Co), their excessive intake engenders adverse health effects [8, 34, 248,

249]. The toxicity mechanism of heavy metals and their ions generally involves entering the organism's body via various paths and interrupting cellular functions by biologically binding to molecules such as proteins or enzymes, thereby creating toxicity inside the human or animal body [8, 248, 254]. Appendix C-1 summarizes the commonly known heavy metal ions, their paths of release, their maximum permissible levels, and their effects on the environment and living beings.

Among these, Cu^{2+} (permissible level in water: 1.3 mg/l (USEPA)[248], 2 mg/l (PUB)[255]) may present excessively in water bodies due to its heavy use in the electrical and electronic industry, the construction industry, and so forth and lead to neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease [34, 256-258]. In addition, Ni^{2+} (permissible level in water: 0.03 mg/l (USEPA)[249], 0.07 mg/l (PUB)[255]) is known to be carcinogenic and a cause of fatal cardiac arrest and impaired lung function and may enter environmental water through Nickel plating, manufacturing alloys, or the battery industry [249, 259-261]. Further, previous literature shows the presence of these ions in the aqueous phase in concentrations that exceed the permissible levels indicated by the authorities [249, 262], and since the use of ICP for the preconcentration of heavy metal ions, such as Cu^{2+} and Ni^{2+} , which are considered toxic micropollutants, on a μPAD has, to the best of my knowledge, not yet been studied, this study aimed to preconcentrate Cu^{2+} and Ni^{2+} , as the analytes of interest, with the aid of ICP on a μPAD .

5.2 Working Principle

ICP is an electrokinetic transport phenomenon observed near perm selective nanoporous membranes. The membranes consist of non-engineered nano pores, in which the dimensions are typically comparable to the thickness of the electric double layer. Given that the membranes comprise uniform surface charges, at sufficiently low electrolyte concentrations (<10 mM), the EDL reaches a thickness of a few nanometres. When the EDL exceeds half-diameter/thickness of nano pores, they overlap in nanoporous structures, leading counter-ions (ions with an opposite charge to the nano pore surface charge) to prevail in pores, driving out co-ions (ions with a similar charge to the nano pore surface charge) from the membrane. In such a scenario, counter-ions are allowed to pass through the membrane due to its perm selectivity, while co-ions are repelled. The total ion flux $J^\pm$ through the membrane, which is affected by both diffusion due to the concentration gradient (∇c) and the applied electric field, can be expressed as follows:

$$J^\pm = J_D^\pm + J_{EP}^\pm = -D_\pm \nabla c^\pm - c^\pm \mu_\pm E \quad (5.1)$$

where $D_\pm$, $c^\pm$ and $\mu_\pm$ stand for the diffusivities, the concentration of the ions, and the electrophoretic mobility of the ions, respectively. This explains the non-zero flux of counter-ions and the near zero flux of co-ions through the membrane, which creates ion depletion and ion enrichment zones at either side of the membrane to maintain the electroneutrality of the system. Eventually, a concentration band can be created at the edge of the depletion zone as the system imbalance influences both counter- and co- ions. As the perm selectivity of AEMs differs from that of CEMs, however, ion depletion occurs at the cathodic and anodic side for the two membranes, respectively. Hence, the

membranes also differ in terms of the intensity of ion depletion, which depends on the diffusivities of the cations and anions present in the system, that is, considering a CEM (AEM), the current through the CEM (AEM) is completely carried by cation (anion) flux, while this only contributes partially to the current in the bulk solution. This imbalance in charge carriers causes depletion of ions, which can be explained by the transference number ($t^\pm$), a function of the diffusivities of ions.

$$t^\pm = D_\pm / (D_+ + D_-) \quad (5.2)$$

Thus, despite the nature of the perm selectivity of the membrane, ion depletion is determined by the amount of co-ions present in the system, and its intensity is determined by the diffusivity of the co-ions, that is, the higher the diffusivity of the co-ions, the stronger the formation of the depletion zone near the nanoporous membrane [62, 72, 73, 102]. Fig. 5.1 presents a schematic illustration of ICP near both the CEM and the AEM.

Heavy metal ions are cationic in nature, and their diffusivities change from metal to metal. Appendix C-2 provides a summary of the diffusivities of different heavy metals and other ions. Further, the anions present in the system also play an active role. These properties can collectively support the preconcentration of heavy metal ions by implementing ICP.

Figure 5.1: Schematic illustration of the ICP phenomenon at a perm selective membrane (A) Distribution of ion concentration at either side of a CEM, (B) Distribution of ion concentration at either side of an AEM, J^+ and J^- are cation and anion flux respectively. The subscriptions $_D$ and $_{EP}$ stand for diffusion and electrophoresis.

5.3 Experimental Methods

5.3.1 Device Design and Fabrication

Two devices were designed for the use of Nafion 212 (CEM) and FAS 50 (AEM) as perm selective membranes, separately. Both devices comprised a simple straight channel and four major layers prepared using a custom electronic cutter and respective design software (Silhouette America, Inc., Silhouette Cameo).

The bottom paraffin layer was assembled with the adjacent paper channel composed of Whatman No. 1 quantitative filter paper by heating it at 110°C in an oven and then cutting it into the final shape of the device. The positioning of either the Nafion 212® or the FAS 50 membrane was then done on top of the paper channel at a pre-determined distance from the anode: 15 mm for Nafion 212 and 5 mm for FAS 50. Next,

assembly of the microwire electrodes of the device was performed: anode: sample reservoir (Nafion 212), buffer reservoir (FAS 50); cathode: at the edge of the membrane (Nafion 212), sample reservoir (FAS 50). A top paraffin layer cut in the shape of the device with openings for sample and buffer feeding at the reservoirs was then used to cover the paper channel assembly and clamped between two glass slides secured with Al foil and heated for 3 min at 110° C. Fig. 5.2 illustrates the descriptive device geometries and configurations of the devices for both Nafion 212 and FAS 50.

Figure 5.2: Schematic of device geometry and configuration (A) Device geometry for the μ PAD that comprises the Nafion 212 membrane, (B) Device geometry for the μ PAD that comprises the FAS 50 membrane.

5.3.2 Material and Chemicals

Whatman No. 1 filter paper purchased from United Scientific, Singapore, off-shelf Nafion® 212 and FAS 50 membranes purchased from Fuel Cell Store, Texas, and laboratory paraffin film (Parafilm M) were employed during the fabrication of the devices. CuCl_2 , $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, and Na_2SO_4 were purchased from Sigma–Aldrich. Calcium Green-5N (CG5N), Hexa potassium salt, and cell impermeant ($\text{C}_{43}\text{H}_{26}\text{Cl}_2\text{K}_6\text{N}_4\text{O}_{18}$) were purchased from ThermoFisher Scientific (Singapore). PBS buffer solution was purchased from Vivantis Technologies Sdn. Bhd. (Malaysia). Ultra-

pure type-01 de-ionized water was obtained from a Direct-Q 5UV water purification system.

Stock solutions of 0.1M were prepared for all the purchased chemicals, except for CG5N, using ultra-pure type-01 de-ionized water. A 40- μ M solution of CG5N was prepared directly as the stock solution. Later, CuCl_2 , $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, and $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ solutions were diluted to make 40- μ M solutions for the experiments. $\text{CuCl}_{2(\text{aq})}/\text{NiCl}_{2 \cdot 6\text{H}_2\text{O}(\text{aq})}/\text{CuSO}_{4 \cdot 5\text{H}_2\text{O}(\text{aq})}$, CG5N, and $\text{PBS}/\text{Na}_2\text{SO}_4$ were mixed in a ratio of 1:1:2, according to the given order of chemicals, prior to the experiments to further dilute the analyte of interest (Cu^{2+} , Ni^{2+}), to allow for bonding between CG5N and the analyte of interest, and to ensure a sufficiently high background electrolyte concentration (for CuCl_2 , $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, PBS; for $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, Na_2SO_4). During calibration of the fluorescence intensity, the same ratio of mixing was followed by changing the concentration of both the analyte of interest and CG5N, except for the background electrolyte.

5.3.3 Experimental Setup

The experimental setup was composed of an inverted microscope (Nikon Eclipse TE2000 – U), a high-speed complementary metal oxide semiconductor (CMOS) camera (Phantom Miro eX4) capturing the images, a high-voltage power supply (Stanford Research Systems, Inc., model PS350), and a computational interface for data acquisition and analysis. Customization of the experimental setup was performed as required by the experiments, where necessary. The analysis of the data was carried out mainly using “ImageJ”.

5.4 Results and Discussion

5.4.1 Characterization of μ PAD for Heavy Metal Ion Concentration

As the characteristics of ICP performance differ depending on the analyte of interest and the device geometry/channel thickness, characterization experiments were conducted, primarily to identify the working electric potential range.

5.4.1.1 *Working Electric Potential Range*

ICP is performed under the presence of a DC electric field. Upon creation of the depletion zone, the current versus the applied voltage behaves in such a way that no significant increment in the current occurs, even though the applied voltage is increased up to some point. This region of voltage is known as the limiting current region and happens to be the working electric potential range to implement ICP. The lower and upper limits of voltages for this region differ depending on system parameters such as electrolyte concentration, channel geometry, types of ions present in the system, and the type of perm selective membrane involved. Hence, we studied the current-voltage ($I - V$) relationship for both Nafion 212 and FAS 50 membranes for the same channel thickness.

The $I - V$ curve observed with the involvement of Nafion 212 shows the Ohmic region, limiting current region, and over-limiting region following the typical pattern of an $I - V$ curve for ICP as the applied voltage is gradually increased. As for FAS 50, even though these regions can be distinguished, the limiting current region behaves differently than it does in the conventional curve. Here, the current shows near zero change up to some voltage and then reduces with increasing voltage; however, ICP can still be

implemented in this region. This can be explained by the diffusive transport of ions that occurs against the applied electric field to support the creation of a concentration band at the cathodic side when an AEM is incorporated in the system. Hence, the flux of ions in the direction opposite to the applied electric field reduces the total current carried in the system. As the voltage is further increased, the current increases again, giving rise to the over-limiting region, where electro convection is dominant and the depletion zone is no longer observed. Fig. 5.3A illustrates the I – V curve for Nafion 212, while the I – V curve for FAS 50 is illustrated in Fig. 5.3B.

Figure 5.3: Current versus voltage relationship for the devices fabricated using Nafion 212 and FAS 50. (A) I – V curve for the device with Nafion 212. Sample solution used was CuCl_2 :CG5N:PBS mixed in 1:1:2 ratios by volume. (B) I – V curve for the device with FAS 50. Sample solution used was CuSO_4 :CG5N: Na_2SO_4 mixed in 1:1:2 ratios by volume. Shown in the dotted lines are (I) the Ohmic region, (II) the Limiting region, and (III) the Over-limiting region of voltages.

5.4.1.2 Preconcentration of Heavy Metal Ions Using ICP

Preconcentration of Cu^{2+} and Ni^{2+} Using Nafion 212 as the Perm Selective Membrane

Nafion is known to be used as an ion exchange membrane for metal ion adsorption, as negatively charged sulfonic groups in the Nafion membrane allow uptake of metal ions, which are cations [263, 264]. Upon application of the electric field in limiting the current regime, however, ICP can be implemented to preconcentrate these metal ions.

Hence, once the device is characterized, ICP is performed initially to the Cu^{2+} aqueous solution made using CuCl_2 , CG5N, and PBS as described in section 3.2. Upon dilution by mixing with CG5N and PBS, the concentration of Cu^{2+} in the solution is 10 μM . The selected voltage for ICP is 70 V, according to the I – V curve. During the implementation of ICP, it showed a clear increment and decrement in fluorescence at the concentration band in a repetitive manner. Fig. 5.4A shows the repetitive increment and decrement of fluorescent intensity with time and the respective variation of current with time. As the fluorescence intensity surpasses the intensity observed at the earlier incremental phase in the next one, this decrement cannot be attributed to the channel saturation. The possible scenario is to consider the counter-ionic behaviour of Cu^{2+} in the presence of Nafion 212, a CEM that initially allows the passage of cations such as Cu^{2+} , before the depletion zone is formed [73]. In this way, as the concentration of Cu^{2+} increases near the depletion zone, there might be a tendency to transport Cu^{2+} through the membrane due to electric double layer destruction inside the nanoporous membrane, and once the EDL is reformed and the double layers overlap, the concentration of Cu^{2+} increases again, forming the concentration band. A similar phenomenon is observed when

ICP is performed using a solution of Ni^{2+} prepared using NiCl_2 , but it is observed at a delayed time (Fig. 5.4B). This delayed response can be attributed to the lower diffusion co-efficient and lower affinity of Nafion towards Ni^{2+} , which are lower than those for Cu^{2+} [265, 266]. In previous literature, Nasef et al. reported the lower affinity and reduced metal uptake of Nafion 117 towards Ni^{2+} compared to Cu^{2+} [263]. Appendix C-2 summarizes the diffusion co-efficients of some of the well-known cations and anions. During the present study, we observed a similar behaviour from Nafion 212 towards Ni^{2+} , showing a lower affinity. Further, since the diffusion co-efficient of Ni^{2+} is slightly lower than that of Cu^{2+} , the movement of Ni^{2+} towards Nafion is hindered. This allows Ni^{2+} to be concentrated in the presence of Nafion for a longer time than Cu^{2+} , despite its counter-ionic nature, before the repetitive increment and decrement are observed.

To investigate the possible scenario of counter-ion transport through the Nafion membrane while ICP is performed, the current through the membrane was studied for both Cu^{2+} and Ni^{2+} . To identify the changes in conduction current with the change in the fluorescence intensity, the results are plotted against time in the same frame as for the fluorescence intensity. According to the graphs, the conduction current (current through the membrane) shows an increment where the fluorescence intensity decreases. For Cu^{2+} (Fig. 5.4A), an increment in the conduction current from 95 s to 200 s where the fluorescence intensity decreases, and the current decreases afterwards as the fluorescence intensity also increases. Beyond 600 s, the increment in current as the fluorescence intensity decreases is not as significant, and this can be attributed to the membrane saturation with ion uptake [263]. A further decrement in the conduction current, however, is visible with a further increment in the fluorescence intensity. This suggests a reduction

of the conduction current through the membrane, which is carried by cations (for CEMs such as Nafion) as the Cu^{2+} becomes further concentrated at the anodic side of the membrane.

Figure 5.4: Implementation of ICP for the preconcentration of heavy metal ions, using CG5N as a fluorescent indicator, where Nafion 212 acts as the nanoporous membrane. (A) Fluorescence intensity and conduction current through the membrane versus the time for Cu^{2+} while ICP is implemented. (Top – figurative illustration for fluorescent increment, bottom – graphical illustration). (B) Fluorescence intensity and conduction current through the membrane versus time for Ni^{2+} while ICP is implemented. (Top – figurative illustration for fluorescent increment, bottom – graphical illustration). (C) Calibration of fluorescence intensity versus concentration for Cu^{2+} ions. (D) Calibration of fluorescence intensity versus concentration for Ni^{2+} ions. (Note that the fluorescence intensity values are not observed at the same exposure times for Cu^{2+} and Ni^{2+}).

For Ni^{2+} , a similar trend of conduction current increment through the membrane is observed (Fig. 5.4B) in parallel to the decrement of fluorescence intensity at a delayed time, which is attributable to a lower diffusion co-efficient and the lower affinity of Nafion towards Ni^{2+} , as explained earlier. The conduction current decreases again as the fluorescence intensity increases, following the repetitive nature of increment and decrement in the fluorescence intensity.

This confirms the phenomenon of Cu^{2+} and Ni^{2+} transport/diffusion through the membrane while ICP is implemented, leading to the repetitive concentration and dissipation of ions at the concentration band. Nevertheless, ICP can be implemented upon identifying these conditions to concentrate heavy metal ions when a Nafion membrane is incorporated in the system to implement ICP. For an example, for Ni^{2+} , the effect of diffusion through the membrane is minimal up to a considerable time.

Pre-concentration of Cu^{2+} Using FAS 50 as the Perm Selective Membrane

Due to the repetitive nature of the concentration of Cu^{2+} and Ni^{2+} using Nafion 212, we studied FAS 50, an anion exchange membrane, as the perm selective membrane for the concentration of heavy metal ions by ICP. Cu^{2+} was used as the model ion in this experiment. CuSO_4 , however, was selected instead of CuCl_2 as the source of Cu^{2+} in this case, as the diffusivity of the co-ions should be higher than that of the counter-ions for the successful implementation of ICP. For FAS 50, Cu^{2+} acts as co-ions, and the diffusivity of Cl^- is larger than that of Cu^{2+} , which does not enable successful implementation of ICP, although it is a good choice in the presence of Nafion. Hence, we changed both the background electrolyte and the Cu^{2+} source to Na_2SO_4 and CuSO_4 , respectively,

introducing SO_4^{2-} to the system, an anion in which the diffusivity is less than the diffusivity in Cu^{2+} and Na^+ .

Figure 5.5: (A) Illustration of fluorescent increment with time for Cu^{2+} , using FAS 50 as the nanoporous membrane (Top: figurative illustration, Bottom: Graphical illustration), (B) Calibration of fluorescence intensity versus concentration for Cu^{2+} . SEM images for (C) Nafion 212, (D) FAS 50 membranes showing the porous structures of membranes under an SEM (Magnification: 30,000)

Fig. 5.5A presents the figurative and graphical illustration of the fluorescence increment with time for Cu^{2+} when ICP is implemented using FAS 50. This provides a continuous increment in fluorescence, indicating the continuous concentration of Cu^{2+} at the cathodic side of the membrane until the channel is saturated. Hence, it is evident that

the bottleneck of the repetitive nature of fluorescence increment and decrement is avoided in this case, with the aid of an AEM where the ions of interest, Cu^{2+} , act as co-ions to the nanoporous membrane.

The resulting concentration factor for Cu^{2+} was 10-fold within 15 minutes, which is higher than that observed for the case using Nafion. It is clear that both the concentration factor and the concentration time increased here. This was due to the continuous concentration allowed by the FAS 50 membrane; the dense structure of the FAS 50 membrane may also have affected this. Compared to Nafion 212, FAS 50 is dense in structure, where its porosity is seemingly lower than that of Nafion 212. Hence, membrane saturation may occur rapidly, which in turn results in better depletion and a highly concentrated sample plug. Fig. 5.5C and 5.5D present the SEM images obtained for the two nonoporous membranes, Nafion 212 and FAS 50, respectively.

5.5 Conclusion

In this study, the focus was to study ICP as a rapid and high-sensitivity detection method for aqueous heavy metal ions, as the current commercial methods available are highly complex and time-consuming and require specific technical knowledge. ICP addresses most of these disadvantages, and further, ICP can be implemented on a μPAD , with the aim of minimizing the cost and maximizing the ease of use.

Thus, the use of ICP on a μPAD is shown here as a viable option for the preconcentration of heavy metal ions. The method acts as a simple and a rapid technique to preconcentrate these samples. With the aid of this device, concentration factors of 5-

fold and 8.8-fold were observed for Cu^{2+} and Ni^{2+} , respectively, using Nafion 212 as the nanoporous membrane. The shortcoming of using Nafion 212 is also shown here, whereby it engenders the destruction of the depletion zone and, thus, dispersion of the concentration band repetitively. We demonstrated that this is due to the passage of ions of interest through the membrane, using the conduction current through the membrane. We also demonstrated that the use of a FAS 50 membrane overcomes this bottleneck and enables the continuous concentration of heavy metal ions, leading to higher concentration factors. Here, a 10-fold concentration factor was obtained for Cu^{2+} ions. Further, we studied the I-V curves for both Nafion 212 and FAS 50 and found that for an AEM, that is, FAS 50, the I-V curve shows a reduction of current in the limiting region after a period of constant current due to the flux of ions in the direction opposite to the applied electric field. This method proved a viable option to be applied in the rapid detection of aqueous heavy metal ions.

Antibiotics can be identified as another type of molecule which has been recognized as a potential micropollutant recently. So far, there are no reported methods for rapid detection of these complex molecules. Hence, in the next section, the study is focus on application of ICP for the detection of these complex molecules considering their complex functionalities.

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CHAPTER 6 PRECONCENTRATION OF AQUEOUS ANTIBIOTICS ON A MICROFLUIDIC PAPER BASED DEVICE BY ION CONCENTRATION POLARIZATION

Until recently, antibiotics were not considered an environmental pollutant, even though these are released continuously into the environment. Recent investigations, however, have attracted researchers' attention to the issue that antibiotics are likely to act as persistent environmental pollutants [20, 267-270]. Therefore, in this study, ICP was investigated for the detection of lower concentrations of aqueous antibiotic residues through the preconcentration of these compounds on a μ PAD. Amoxicillin sodium was chosen as the analyte of interest during the study, as it is one of the most heavily prescribed antibiotics around the globe and its presence in water bodies has been detected in studies.

6.1 Introduction

Antibiotics are an important group of pharmaceuticals that are biologically active and highly effective at low dosages against diseases [20, 267, 270, 271]. The molecular weights of these compounds are usually lower than 1000 Da [268]. These are categorized, according to their chemical structure and mechanism of action, into different classes, such as β -lactams, quinolones, and tetracyclines [268, 270]. A detailed list of classes of antibiotics is provided in appendix D-1. Due to the complexity of their chemical structures, these compounds may show cationic, anionic, or neutral behaviours at different pH values [268].

6.1.1 Occurrence of Antibiotics in Nature

Since antibiotics are designed to treat or prevent diseases by acting upon pathogens, that is, through antimicrobial activity, these compounds exhibit some persistence in the living body [270, 271]. Inside the living body, these compounds are not fully consumed, and a considerable portion is excreted into the environment in urine or faeces as the original drug or its metabolites [268-272]. It is estimated that 30% to 90% of the consumed drug is excreted as active compounds and some of the metabolites excreted are more toxic than their parent compound. Further, industrial effluents from antibiotic manufacturing, hospital waste water, and the extensive use of antibiotics as human/veterinary medicine, growth promoters for livestock, aquaculture, and plant agriculture significantly contribute to the presence of antibiotics in the environment [20, 267-269].

The incomplete degradation and incomplete removal at sewage treatment plants (STP) or waste water treatment plants (WWTP) lead these antibiotics to end up [267-269, 271, 272] in various phases of water bodies, such as surface or ground water, and sediments, rendering them ubiquitous in aquatic environments. This occurrence is also supported by metabolites that convert back into the active compound after excretion [267, 270, 273] and by the application of manure or sewage sludge as fertilizer and the use of waste water as a watering source in agricultural fields [267, 269]. A survey in Hyderabad, India, an area of intense pharmaceutical manufacturing, reported that the concentrations of antibiotics in the water from nearby lakes and wells exceeded the concentrations of antibiotics in human therapeutic blood plasma [274].

These enter ground water aquifers used as drinking water sources through leaching and the use of surface water in ground water recharge [267, 269, 270, 272]. Thus, if not treated during the production of drinking water, they enter human/animal bodies and cycle through the food chain. According to the literature, the number of antibiotic compounds detected in wastewater influent/effluent, surface water, ground water, and drinking water exceeds 30 [270]. Further, in 1999, antibiotic N-(phenylsulfonyl)-sarcosine propyphenazone was detected in tap water in Berlin [272].

6.1.2 Micropollutant Nature of Antibiotics

The major risk associated with the presence of antibiotics in aquatic environments concerns their ability to induce or increase the prevalence of antibiotic resistance among various bacterial strains. The higher the concentrations of antibiotics, the higher the resistance of bacteria, and the existence of these antibiotic-resistant microorganisms renders available drugs ineffective against infections [9, 267, 269, 275]. This may lead to epidemic diseases, and it is significant because more than 70% of microorganisms are resistant to at least one antibiotic, while many show multiple resistances [267]. While some microorganisms arguably exhibit primary resistance towards antibiotics, secondary resistance, acquired through exposure to antibiotics, is the main concern. This secondary resistance is transferrable from one organism to another through plasmids and/or genetic material. To further complicate the issue, microorganisms are highly adaptable and reproduce at a rapid pace, engendering genetic mutations in the next generation that enable adaptability to antibiotics [9].

Entry of these microorganisms into the food chain (e.g., vancomycin-resistant enterococci (VRE) and multi-resistant *pseudomonads*) can alter the genes of important intestinal bacteria and create antibiotic resistance in humans/animals [9, 267]. This leads to changes in the human/animal body's nutritional uptake and a reduction in the body's immunity to infectious diseases. Further, antibiotics can play a role in inhibiting and altering environmentally important organisms, such as nitrifying bacteria in surface waters, which convert toxic ammonia to nitrate, and some types of algae that are highly sensitive to antibiotics—such as amoxicillin, benzyl penicillin, and tetracyclines [9, 269]—causing an imbalance in natural microbial communication and, hence, in nature. Antibiotics can also affect the chloroplast replication in plants, causing alterations in the reproduction of some organisms, such as *Daphnia magna*, in their early stage of life, exerting adverse effects on organism populations [9].

Considering these adverse effects, the evidence on discovering lower concentrations of antibiotics in tap water in some regions in the world, indicating the inability of in-line monitoring to detect lower concentrations of antibiotics at the discharge from drinking water production plants, implies the need for rapid and continuous detection/monitoring methods to determine the antibiotic concentrations in aquatic environments. Further, since the concentrations found and expected to be found are usually in lower orders of magnitude, the preconcentration of samples is often required prior to analysis to render them compatible with the limits of detection of analysis equipment.

6.2 Working Principle

6.2.1 Potential of Forming a Polar Antibiotic-Fluorescent Detecting Agent Complex

During the study, an aqueous solution of Amoxicillin sodium was mixed with an aqueous solution of Rhodamine B in a 1:1 (V/V) ratio. The complex formed exhibited negative polarity at a pH of 7.

As the complex formed exhibited a negative polarity, it initially moves forward through the channel due to hydrodynamic forces. Upon subjection to the DC voltage provided along the channel, the complex experiences electrophoretic flow towards the anode, while electroosmotic flow acts in the direction of the electric field, as paraffin has a negative surface charge. The presence of the Nafion membrane restricts the flow of the sample due to the depletion force. Therefore, a concentration of the complex formed was observed on the anodic side of the device as a localized concentration band. Fig 6.1 illustrates the working principle on a fabricated μ PAD.

Figure 6.1: Working principle of implementing ICP on a μ PAD for the pre-concentration of an Amoxicillin sodium–Rhodamine B complex. EOF- Electroosmotic force, F_{Ca} – Capillary force, F_{ICP} – Depletion force from membrane, EP – Electrophoretic force.

6.3 Experimental Methods

6.3.1 Device Design and Fabrication

Based on the experimental goals, μ PADs of three different geometrical configurations were fabricated, following the direct cutting and heating method developed in Chapter 3. The three geometrical configurations were (i) circular reservoir ($\phi = 10$ mm) connected to a short straight channel ($1\text{ mm} \times 7\text{ mm}$) for the purpose of the calibration of fluorescence intensity, (ii) straight channel ($1\text{ mm} \times 20\text{ mm}$) with a sample port ($\phi = 6$ mm) in the middle of the device and two circular reservoirs ($\phi = 10$ mm) at either end of the channel for the study of the polarity of the complex formed, and (iii) straight channel ($1\text{ mm} \times 15\text{ mm}$) connecting to two circular reservoirs ($\phi = 10$ mm) at either end and composed of a Nafion membrane positioned 9 mm from the sample inlet reservoir to study the effect of ICP on the preconcentration and detection of antibiotics. Fig. 6.2 illustrates the device configurations used in this study.

6.3.2 Sample Preparation

A 10 mM aqueous Amoxicillin sodium solution was prepared, and a series dilution was performed to obtain Amoxicillin sodium solutions of concentrations varying from 10 mM to 10 nM. Rhodamine B aqueous solutions were prepared by following the same procedure to yield a series of solutions from 10 mM to 10 nM. This series of solutions served the purpose of calibrating fluorescence intensity. A 0.15-M NaCl solution was prepared, using stock solution of NaCl, to use as a background electrolyte. A 100- μ M Fluorescein sodium solution was also prepared for the study of suitable fluorescent detectors. For the implementation of ICP, an Amoxicillin sodium solution with an initial

concentration of 100 μM was mixed with 100 μM Rhodamine B and 0.15 M NaCl in equal volume fractions, diluting the final concentration of Amoxicillin sodium in the solution to 33.33 μM . Amoxicillin sodium (MedChem Express, USA) was purchased from Bio.Etc Pte Ltd., Singapore. The Rhodamine B, Fluorescein sodium, and Sodium chloride (NaCl, ACS reagent, $\geq 99.0\%$) were from Sigma–Aldrich. All the reagents were prepared using ultrapure type 1 water derived from a Direct–Q 5UV water purification system.

Figure 6.2: Geometrical configurations of the designed μPADs . (A) Geometry used for the calibration of fluorescence intensity. (B) Geometry of the device used for the study of the polarity of the complex formed. (C) Geometrical configuration of the μPAD used for the implementation of ICP.

6.3.3 Experimental Methods

ICP was implemented using a setup similar to the one described in Chapter 3 and Chapter 4, with the device illustrated in Fig. 6.2C. A voltage of 75 V was used upon

studying the current versus voltage relationship, as described in chapters 3 and 4, to identify the limiting region of voltages that could be applied for the purpose of preconcentration using ICP. The fluorescence enhancement of the complex during the implementation of ICP was captured using a highspeed Phantom Miro series camera and the respective software (Phantom Camera Control 2.7). To calculate the concentration factor that would be observable using ICP, the captured frames were analysed using ImageJ for fluorescence enhancement, and the concentration factor was later calculated.

6.4 Results and Discussion

6.4.1 Fluorescent Detecting Agents for Antibiotics

During the study, special attention was paid to the fluorescent detectors for Amoxicillin, as most of the fluorescent probes reported are at the research stage. The literature, however, suggests carbon nanodots as a promising fluorescence-detecting agent for Amoxicillin [37, 276, 277]. Still, the resulting complex is highly stable and nonpolar; hence, it is not suitable for the implementation of ICP. Therefore, several fluorescence detectors, such as Fluorescein sodium salt ($C_{20}H_{10}Na_2O_5$) and Rhodamine B ($C_{28}H_{31}ClN_2O_3$), were explored in terms of their suitability for the application. Initially, only the concentration of the Amoxicillin sodium was changed, and the concentration of Fluorescein and Rhodamine B was kept constant to identify the detection capabilities of these fluorescent dyes.

It was evident that both Fluorescein sodium and Rhodamine B showed reasonable detection capabilities for varying concentrations of Amoxicillin within the solution. Further, it was found that while Fluorescein exhibited better luminance and detection

capabilities, it did not behave as a polar complex once the complex was formed. Hence, it becomes a poor choice for implementing ICP. Rhodamine B, however, did show polarity upon binding with Amoxicillin Sodium salt while showing considerably acceptable detection capabilities. Hence, it can be stated that Rhodamine B is a reasonable choice for Amoxicillin as a fluorescence-detecting agent during implementation of ICP. Fig. 6.3 presents the calibration curves observed for both Fluorescein and Rhodamine B respectively.

Figure 6.3: Calibration curves obtained for (A) Amoxicillin- Fluorescein complex, (B) Amoxicillin-Rhodamine B complex.

This emission of fluorescence can be explained by the potential bonding between Amoxicillin and the fluorescent-detecting agent used, which forms a chemical complex. Further, the differences in emission were due to the varying concentrations of Amoxicillin in the test samples, which changed the active binding sites within the solution, thus changing the extent to which complex formation occurred.

6.4.2 Polarity Characteristics of Antibiotic- Detecting Agent Complexes

To determine the polarity of the complex, an experiment was conducted, using the device configuration given in Fig. 6.2B. The reservoirs at either end were wetted, using 20 μL of NaCl solution. Once the channel was fully wet, 40 μL of complex solution was added to the sample port in the middle. Subsequently, a voltage of 100V was applied, and the movement of the complex either towards the anode or the cathode was observed over time to determine the polarity of the complex.

Upon observing the movement of the complex formed between Amoxicillin and Rhodamine B, it could be seen that the fluorescence intensity at the anodic side increased with time, indicating the negative polarity of the complex. This polar complex formation could be attributable to the hydrogen bonding between the Amoxicillin sodium and Rhodamine B molecules. The structure of Amoxicillin sodium is depicted in Fig. 6.4A, while Fig 6.34B illustrates the structure of the Rhodamine B molecule. The oxygen, nitrogen, and hydrogen atoms in these molecules can act as active sites to form hydrogen bonds between these two molecules and thereby form a chemical complex. The bonding ratio of these molecules, however, is still unclear, since the chemistry underlying complex formation is complicated. Still, the complex formed is negatively charged, and this enables the use of ICP for the preconcentration of this complex, which in turn enables the detection of lower concentrations of Amoxicillin in an aqueous phase.

Figure 6.4: Chemical structure of (A) Amoxicillin Sodium, Source: National Center for Biotechnology Information. PubChem Database. Amoxicillin Sodium, CID=23663126, <https://pubchem.ncbi.nlm.nih.gov/compound/Amoxicillin-sodium> (accessed on 26 Dec. 2019), (B) Rhodamine B, Source: National Center for Biotechnology Information. PubChem Database. Rhodamine B, CID=6694, <https://pubchem.ncbi.nlm.nih.gov/compound/Rhodamine-B> (accessed on 26 Dec. 2019).

Further, to study the stability of this polar complex, the polarity was studied at different pH values (acidic - pH =1, basic – pH = 13). HCl was used to make the acidic solution, and NaOH was used to make the basic solution. Both the HCl and NaOH were obtained from Sigma-Aldrich. Fig 6.5 graphically illustrates the fluorescent intensity emitted by each reservoir of the device over time. It was observed that the fluorescent intensity emitted by the anodic reservoir tended to increase over time whereas cathode and sample reservoir fluorescence remained almost unchanged. Hence, at a neutral pH, however, the complex become negatively polar; thus, ICP can be performed conveniently. Still, further studies should be conducted to identify the nature of the complexes formed upon binding with the fluorescent probes used in this study. Also, the stability of polarity of the complex formed should be studied at different pH values to enable preconcentration of antibiotics as most of the antibiotics demonstrate a negatively polar structure at higher pH.

Furthermore, a deposition of a black-coloured compound was observed for each pH at the anodic reservoir. Hence, the fluorescent measurements for the anodic reservoir fluctuated somewhat, rendering it difficult to reach a conclusion regarding the polarity of the complex. However, as cathodic and sample reservoirs' fluorescence intensity was kept almost unchanged, the polarity of the complex was concluded to be negative. Even though most of the antibiotics become negatively polar at higher pH, considering that the water commonly available for human use fluctuates around the midpoint of the pH scale, the sample solution of neutral pH was used for the further experiments.

Figure 6.5: Fluorescence intensity emitted by Amoxicillin-Rhodamine B

6.4.3 Study of Working Electric Potential Range

Applied voltage is a critical factor in performing ICP for preconcentration. The conventional curve for current versus voltage for ICP comprises three regions: (I) the Ohmic region, where the current linearly increases with applied voltage until the nanoporous membrane becomes saturated due to the transportation of ions through it, (II) the limiting region, which is created as a result of membrane saturation and where the current remains approximately constant up to some point, when the voltage increases, and (III) the over-limiting region, which is observed under considerably high voltages due to the electroconvection destroying the depletion zone. Of these regions, ICP can be implemented for preconcentration and detection purposes only in the limiting region. Hence, a current-versus-voltage study was conducted to identify the working electric potential range.

During the present study, it was observed that a working voltage within a range of 40V to 75V would be ideal for conducting the experiments. Therefore, the experiments were conducted within this range to determine the optimum applicable voltage, and 75V was found to represent the best compromise between the ICP performance and the stability of the reaction. Hence, 75V was used as the working electric voltage during implementation of ICP. A consistent ICP band can be obtained by using the aforementioned voltage.

Figure 6.6: Current-versus-voltage curve for the designed device for the performance of ICP. Labelled are (I) the Ohmic region, (II) the limiting region and (III) the over-limiting region.

6.4.4 Implementation of ICP for Detection of Antibiotic in an Aqueous Phase

This study was conducted using the geometry given in Fig 6.2C, applying a voltage of 75V for the samples prepared as discussed in section 6.4.2. The initial concentration of Amoxicillin in the test sample was 33.3 μM . A rapid concentration of Amoxicillin-Rhodamine was observed, giving rise to a clear concentration band within a time period as short as 270 s; however, it was observed that the fluorescence intensity emitted peaked at around 180 s and then decreased over time. This was due to the saturation of the paper channel by the sample solution used for the study; this is commonly observed in paper channels.

Fig 6.7 presents the figurative and graphical illustration of fluorescence enhancement over time for the Amoxicillin-Rhodamine B complex at the sample plug,

with the calibration curve of the fluorescence intensity versus concentration observed for the varying concentrations of this complex. Upon the performance of ICP, a 3-fold concentration factor was observed within a short period of time, such as 180 s. This indicates that the performance of device is extremely rapid. Further, the device is inexpensive, disposable, and convenient to use. In addition to this, the concentration at the anodic side of the Nafion membrane during the implementation of ICP itself confirmed the polarity of the complex formed upon mixing Amoxicillin sodium and Rhodamine B, thus demonstrating Rhodamine B to be a suitable fluorescent detector for ICP during detection of Amoxicillin.

Figure 6.7: Preconcentration of the Amoxicillin-Rhodamine B complex on the designed μ PAD. (A) Figurative representation of the fluorescence enhancement over time at the sample plug at the anodic side of the membrane. (B) Graphical representation of the fluorescence enhancement over time at the sample plug for the complex of Amoxicillin-Rhodamine B. (C) Calibration curve showing the fluorescence versus varying concentrations of the Amoxicillin-Rhodamine B complex.

6.5 Conclusion

Antibiotics have recently been identified as a micropollutant that can exert adverse effects on human/animal life and the environment upon their presence in aquatic environments. Some of the adverse effects are the development of antibiotic-resistant microorganisms and the development of antibiotic resistance in the human and animal body, lowering the immunity towards bacterial diseases, and so forth. With the excessive use of antibiotics all around the globe, there is an incremental risk, and attention should be paid to the detection and monitoring of these compounds in aquatic environments.

Therefore, during this study, the focus was placed on the detection of aqueous Amoxicillin, a highly prescribed antibiotic, through the application of ICP on a μ PAD. Amoxicillin has also reportedly been found in water bodies in several places around the globe. During this study, fluorescent-detecting agents for Amoxicillin that are suitable for ICP were studied in detail, as most of the fluorescent-detecting agents for antibiotics are still at the research stage and the complexes formed exhibit nonpolar behaviour. It was found that Rhodamine B is a reasonably effective fluorescent probe for Amoxicillin that can be used during implementation of ICP, as it produces a polar complex upon binding with Amoxicillin at a neutral pH. Further studies, however, should be conducted with respect to pH dependence of polarity of the complexes formed, as most antibiotics show a negative polarity at higher pH values in an aqueous medium, enabling detection using ICP. Hence, fluorescent probes that secure the negative polarity of these antibiotics are of paramount importance for the application of ICP for the detection of antibiotic compounds, where fluorescence detection is applied as the detection method.

The experiments for the implementation of ICP were conducted at a voltage of 75V, as the results for the current-versus-voltage curve indicated. A 3-fold concentration factor was observed within 180 s, enabling rapid detection of lower concentrations of aqueous amoxicillin. Further, this developed device is easy to fabricate, easy to use, and inexpensive. With the use of suitable fluorescent detectors and the embedding of simple software for onsite monitoring, this device can also be optimized for the detection of other types of antibiotics.

Next section of the thesis is allocated for the discussion of the conclusions drawn from studies reported here and to propose recommendations for the future based on the work carried out here and ongoing studies.

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CHAPTER 7 CONCLUSIONS AND FUTURE WORK

7.1 Conclusions

In the present thesis, the fundamental electrokinetic phenomenon of ICP is studied, and its application on a microfluidic scale in detecting analytes in a wider range is investigated, using paper-based microfluidic devices as the platform. Through presenting a novel fabrication method for the fabrication of μ PAD, a disposable, easy-to-use, and hand-held μ PAD was developed for the implementation of ICP and demonstrated better performance, giving higher concentration factors than the previously reported devices. The use of ICP as a preconcentration and detection technique for the monitoring of aqueous micropollutants was studied in detail. A μ PAD was developed for the detection of pathogens in water through the rapid preconcentration of these organisms within 10 mins. A μ PAD was also developed to study the effect of ICP on heavy metal ion detection, and the bottlenecks arising due to polarity, diffusivity, and affinity were carefully addressed to obtain superior performance. Finally, the use of ICP for the detection of complex molecules of antibiotics was studied, and the problems encountered as a result of the complexity of these molecules were addressed. This study provides a better understanding of the ICP phenomenon and its applicability for the detection of a vast range of analytes. The conclusions derived from the study can be summarized as follows.

- 1) First, I have developed a novel, rapid, convenient, and inexpensive fabrication method for μ PADs. It was shown that an encased converging microfluidic paper channel provides better performance in the preconcentration of diluted samples, providing an enhanced concentration factor of 2019. The effects of EOF, sample

evaporation, and channel geometry were studied comprehensively, and it was concluded that a reduced EOF due to the use of hydrophobic substances with lower zeta potential, the minimization of evaporation through the encasement of the paper channel, and the converging-geometry-induced sample squeezing in the converging region collectively contributed to the enhanced sample concentration, resulting in higher concentration factors. In addition, a scaling analysis of depletion length and applied voltage was performed to show the adjustability of the developed design to predefine the concentration zone for different analytes of interest. The findings suggest the potential of the device to be used in a wider range of diagnostic and environmental monitoring applications.

- 2) A μ PAD was developed for the rapid preconcentration of *Escherichia coli*, using the method developed in the first study. The fabricated device enabled preconcentration of *E. coli* with a high concentration factor of 2×10^5 -fold within minutes. The effect of a cell suspension on EOF and the effect of the ionic concentration in the cell suspension media for ICP were studied. It was shown that a cell suspension lowers the EOF through channel, thereby leading to the creation of a denser concentration band. In addition, the working electric potential is reduced with the increment of ionic concentration, and the implementation of ICP is faster in this case. There is an upper limit for this increment, as saturation of the nanoporous membrane engenders saturation of current in the system. Overall, this can be suggested as a rapid and convenient method for detecting *E. coli* that can serve as an alternative to the time-consuming conventional detection methods available for microorganism

detection in ballast water, coastal water quality monitoring, as the water is of high salinity. The device shows the potential to be used in low-salinity water through a simple sample preparation step of ion concentration adjustment.

- 3) Third, attention was paid to the detection of aqueous heavy metal ions, which are highly toxic and are found in tracer concentrations in water. The design of the μ PAD developed by incorporating a Nafion membrane resulted in concentration factors of 5 and 9 for Cu^{2+} and Ni^{2+} , respectively, within minutes. The incorporation of Nafion resulted in a repetitive increment and decrement of fluorescence for both ions, and upon studying this effect, I concluded that this was due to the flow of counter-ions through the membrane due to the destruction of the double layer within membrane pores. To address the issue, a device was developed incorporating a FAS 50, an AEM, and a continuous concentration of ions was observed with a concentration factor of 15. This enabled obstacle-free heavy metal ion detection. Current-versus-applied-voltage variations were also studied for both membranes, and it was observed that the flux of ions in the direction opposite to the electric field when AEM is incorporated leads to a reduction of current in the limiting region, without obstructing the process of ICP.
- 4) To demonstrate the applicability of ICP for the detection of micropollutants, which are complex molecules such as antibiotics, through preconcentration, ICP was performed for an aqueous solution of Amoxicillin sodium on a μ PAD. Here, Rhodamine B was found to be a suitable fluorescent probe for Amoxicillin that enabled the application of ICP for the preconcentration of the polar complex formed. This addressed the void of lack of fluorescent probes that enable both

detection capabilities and the formation of a polar complex. A 3-fold concentration factor was achieved within 180 s, demonstrating the rapid preconcentration and detection capability of the fabricated device. Nonetheless, with the application of the proper detection agents, this device can also be optimized for the detection of other types of antibiotics or complex molecules.

7.2 Future work

Some of the important findings and conclusions reached in the present thesis are discussed above. Based on the preliminary studies conducted and ongoing projects, however, the following projects would be of great relevance as a continuation of the current work discussed in this thesis.

- 1) As demonstrated in the literature review, the phenomenon of ICP is explained theoretically using relevant governing equations, and most of the theoretical studies reported have focused on only one aspect of the phenomenon, such as the over-limiting current [75-77, 80]. The phenomenon lacks a complete theoretical study on preconcentration by ICP, especially for the implementation of this concept on a porous medium such as a μ PAD. The only reported study on this issue was for PDMS channels and was a 1D study, which is not universal for the applications of ICP [69]. Hence, it is of great importance that a theoretical study be conducted, addressing all aspects of ICP on a μ PAD or a porous medium to provide a mathematical model that is universally applicable.
- 2) In chapters 4, 5, and 6, studies are presented on the detection of some selected analytes of various micropollutant categories, such as *E.coli*, Cu^{2+} , Ni^{2+} , and

Amoxicillin. Numerous micropollutants belonging to these categories, however, are present in water. Experiments should be conducted on the simultaneous detection of micropollutants on a single platform, utilizing their distinctive properties, such as surface charge characteristics, magnetic susceptibilities, and activation of functional groups. Hence, it is recommended to continue this study on the simultaneous detection of these micropollutants on a single platform by utilizing the differences between them in terms of their distinguishable properties as a driving force to selectively concentrate them on a single platform while implementing ICP by combining different kinds of force fields, such as electric and magnetic fields.

- 3) In chapter 6, the effect of ICP was investigated for the detection of aqueous antibiotic residues at a neutral pH. The study should be extended, however, to study the stability of polar complexes at different pH as these complex molecules of antibiotics demonstrate polar characteristics at sufficiently low or high pH. It is evident that at a higher pH most of these molecules are negatively polar. Further, the use of electrochemical detection in place of fluorescent detection can potentially address this drawback of stability of polar complexes at varying pH and enable the use of ICP for detection of lower concentrations of aqueous antibiotics. Hence, attention should be paid on this direction to further enhance the use of ICP in the field of environment monitoring.
- 4) In the present study, the experiments were conducted by feeding the device batchwise; however, experiments should be extended to the continuous concentration of samples through optimization of the fabricated μ PADs to

withstand the continuous feed of a sample and the force fields that act collectively on the device. This would allow for studying the effect of combined forces on sample concentration and detection by ICP to better penetrate the performance of ICP on a μ PAD and to optimize the devices to remain robust under extreme conditions of use, such as high pressure, long durations of process, and wettability of solutions. Further, a continuous feed would lead to achieving a lower limit of detection for the device, thus broadening the limits of detection by ICP.

- 5) In this current work, the application of ICP was demonstrated for different types of micropollutants; their characteristics and requirements were analysed, and the difficulties that arose were addressed. The phenomenon shows great potential to be used as a simple onsite monitoring technique for environmental monitoring or diagnostic purposes. Therefore, an onsite monitoring platform of a hand-carriable size, with reduced weight, should be developed. For this purpose, electrochemical detection is recommended as the detection method instead of the fluorescent detection used within the context of this thesis, due to the difficulty of finding light sources of different wave lengths for the excitement of fluorescence. Electrochemical detection can be implemented with the aid of a combination of simple components, such as a low-cost, programmable potentiostat, a fabricated triboelectric generator [172] to provide the electricity required for the potentiostat, and simple, three-electrode screen-printed electrodes. The voltage required for the implementation of ICP could be provided using a stack of portable (12 V/16 V) batteries, following the studies

presented in this thesis to lower the required voltage for ICP, and the μ PAD design should be optimized so that a disposable paper platform that can be placed on a reusable platform designed for the detection of the electrochemical would be fabricated.

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Appendix A

A-1 Calculation of channel thickness

Paper channels with different thicknesses were fabricated as described in material and methods section.

Whatman Grade 1 qualitative filter paper standard thickness = $180\text{ }\mu\text{M}$

Parafilm M paraffin film thickness = $130\text{ }\mu\text{M}$

Analysis was carried out using Image J software and Whatman Grade 1 filter paper thickness was used as a reference in calculation of thicknesses.

Therefore 1 pixel = $0.341716\text{ }\mu\text{M}$

Consider that reduction of thickness is due to penetration of wax into the paper channel.

For the one side paraffin coated device;

Thickness if there is no penetration = Whatman Grade 1 filter paper thickness + paraffin film thickness

Thickness if there is no penetration = $(180 + 130)\text{ }\mu\text{M} = 310\text{ }\mu\text{M}$

Hence, the reduced thickness from 310 can be taken as penetrated paraffin thickness (paraffin layer thickness)

Channel thickness = $180 - \text{wax layer thickness}$

For the both side paraffin coated devices;

Thickness without heating (no penetration) = $(130 + 180 + 130) = \mu\text{M} = 440 \mu\text{M}$

In a similar manner,

Paraffin layer thickness = $440 - \text{measured thickness}$

Channel thickness = $180 - \text{wax thickness}$

Calculated thicknesses following the above method;

Table 1: Thickness of fabricated μPADs depending on the second stage heating time

2 nd Stage heating time (min)	Channel thickness (μm)	Paraffin thickness (μm)
0	177.954	2.046
1	177.436	2.564
2	170.534	9.466
3	151.975	28.025
4	149.848	30.152
5	133.897	46.103

A-2 Cost of fabrication per device

Table 2: Cost of fabrication per μ PAD

Material	Supplier	Product	Unit	Total cost (USD)	Unit cost (USD/cm ²)	Device cost (USD)
Whatman No. 1 chromatography paper	United scientific equipment pte. Ltd.	WH-1001-150	15.0 cm dia 100 sheets/pk	16.04	0.0009077	0.0401
Paraffin film (parafilm M)	SPI supplies	01852-AB	4 in x 250 ft /pk	63.46	0.0008197	0.0451
Nafion perfluorinated membrane	Alfa Aesar (Thermo Fisher Scientific Inc)	676470	30 cm x 30 cm /sheet 1sheet/pk	165.11	0.1834555	0.2220
100-micron copper wire	Sigma Aldrich	GF52128008	100-micron dia 100 m/roll	154.68	0.0154676 (per cm)	0.0464
Device cost (USD) [1 USD = 1.39 SGD] [1 USD = 66.80 INR]						0.3536

A-3 Fabrication time for a single device and batch wise

Table 3: Fabrication time for a single μ PAD and batch wise

Fabrication step	Time taken per device	Time taken per batch (8 devices)
Cut Whatman filter paper, aluminium foil and paraffin film	1 min	3 min
Assembly of these layers	1min	4 min
Heating in oven at 80 °C	5 min	5 min
Cooling down time	3 min	3 min
Cut the device geometry by paper cutter	1 min	7 min
Assembly of top paraffin layer, nafion membrane and copper wire	2 min	16 min
Final heating in oven at 80 °C	3 min	3 min
Time taken for the fabrication per device	16 min (approximately)	6 min (approximately)

A-4 Fluid front movement through paper channel for converging diverging section

A-5 Calculation of EOF velocity on μ PAD

Channel length of μ PAD = 25 mm

Applied voltage = 400 V; this is equivalent to the electric field strength of 1.6×10^4 V/m.

Three devices with different heating times were used during EOF measurement. The calculation below is illustrated based on one side paraffin coated (5 min heated in oven) device.

Time taken to reach the steady current = time taken to travel the distance between two reservoirs by lower concentration solution = 125 s

EOF velocity;

$$EOF \text{ velocity} = \frac{\text{Distance between two reservoirs}}{\text{Time taken to reach steady current}} \text{ --- (1)}$$

$$EOF \text{ velocity} = \frac{25 \text{ mm}}{125 \text{ s}} = 0.2 \text{ mm/s}$$

Similarly, EOF velocity for other devices can also be measured.

Following table summarizes the EOF velocities of all three devices.

Table 4: EOF velocity for μ PADs fabricated with different heating times

Device (heating time)	Time taken to travel 25 mm	EOF velocity
5 min	125 s	0.2 mm/s
7 min	150 s	0.167 mm/s
9 min	190 s	0.132 mm/s

A-6 Calculation of concentration factor

Concentration factor is calculated with reference to the calibration curve prepared at the beginning of the study. In each case of sample pre-concentration fluorescein sample solutions of 40 μM initial concentration which corresponds to fluorescence intensity of 23.3 was used. The calculations are illustrated here for the both side paraffin coated devices.

Initial concentration = 40 μM

Respective fluorescence intensity = 23.3

Maximum fluorescence intensity obtained = 223.412

Corresponding concentration = 80.78 mM

Concentration factor;

Concentration factor

$$= \frac{\text{concentration obtained at the end of implementing ICP}}{\text{Initial concentration}} - - (2)$$

$$\text{Concentration factor} = \frac{80.78 \times 10^{-3}}{40 \times 10^{-6}} = 2019.5 \text{ folds}$$

Appendix B

B-1 Schematic of the device with dimensions

B-2 Fluorescence spectra for SYTO 9

Extracted from Thermofisher spectraviewer (<https://www.thermofisher.com/sg/en/home/life-science/cell-analysis/labeling-chemistry/fluorescence-spectraviewer.html>)

Appendix C

C-1 Occurrence and effects of heavy metal ions

Table 5: Heavy metal ions, their occurrence of nature, permissible levels and their adverse effects

Heavy metal	Speciation	Paths of release	(WHO) permissible level (mg/l)	Effects
Mercury (Hg)	oxidation states: 0, +1 and +2, Most stable: Hg^{2+} , Hg_2^{2+} under oxidizing conditions May occur as methyl/ethyl mercury [248, 278]	Coal burning, volcanic eruptions, Incineration of municipal solid waste, mining and smelting [8, 278, 279]	0.001 [8] 0.002 (USEPA) [248, 252] 0.006 (inorganic form, PUB) [255]	Effects on lungs, kidneys, skin and eye, digestive system, central nervous system, origin of diseases like acrodynia and minamata, causes hypertension [8, 278, 279]
Cadmium (Cd)	oxidation states: 0, +2, Most stable: Cd^{2+} (lower pH <8) [248, 278]	Electroplating, paints, pigments, plastic stabilizers, batteries, photographic process, photoconductors, Cd alloys – Cd-Ag solders, fire detection systems, telephone wires [8, 278]	0.005 [8] 0.005 (USEPA) [248, 252] 0.003 (PUB) [255]	Itai itai disease – (renal dysfunction, skeletal deformation), hypertension, weight loss, lung cancer, osteoporosis, fatigue, chronic anemia [8, 249, 278]
Lead (Pb)	oxidation states: 0, +2, Most common/reactive: Pb^{2+} , Most stable: Pb^{2+} , Pb^{4+} [248, 278]	Mining and smelting, piping for sanitation, combustion of gasoline with lead, paints, pigments, metal products, storage batteries [8, 278, 280]	0.05 [8] 0.015 (USEPA) [248] 0.01 (PUB) [255]	Neuro degenerative diseases, kidney damage, decreased bone growth, resemble Ca in deposition and lead to higher concentrations of lead in bones, penetration through blood brain barrier, neurological, cardiovascular disorders [8, 278, 280]
Arsenic (As)	oxidation states: -3, 0, +3, +5, Common As^{5+} [Arsenate (AsO_4) ³⁻] in aerobic environment, As^{3+} [Arsenite (AsO_3) ³⁻] in reducing	Arsenic based wood preservatives, pesticides, herbicides and insecticides, fertilizers, detergent, poultry feed additive, pharmaceuticals, mining and smelting, oxidation of pyrite	0.01 [248, 252, 282] 0.01 (USEPA) [248] 0.01 (PUB) [255]	Effects on central and peripheral nervous system, gastrointestinal tract, damage to kidneys, causes respiratory distress, cardiovascular and pulmonary diseases, skin cancer [8, 278]

	conditions, Arsine (AsH ₃) is highly poisonous [248, 278, 281]	[8, 278]		
Chromium (Cr)	oxidation states: 0, +3,+6, Common Cr ⁶⁺ [Chromate (CrO ₄) ²⁻ , dichromate (Cr ₂ O ₇) ²⁻] shallow aquifers, Cr ³⁺ at low pH (<4) [248, 278]	Ferroalloy production, leather tanning, refractory bricks, pigments and paints, chrome plating processes, catalysts [8, 29, 278]	0.05 [8, 282] 0.1 (USEPA) [248] 0.05 (PUB) [255]	Lung cancers, dermatitis, carcinogenic, mutagenic and teratogenic, toxic to reproductive system, effects on blood glucose level [8, 29, 278]
Silver (Ag)	oxidation states: +1, +2,+3, Most common Ag ⁺ [283-285]	Electronic, photographic and imaging industry, pharmaceuticals, electroplating, jewelry, refining of copper, nickel, gold, zinc [8, 254, 284, 285]	0.1[8] No guideline [282] 0.05 (USEPA) [285]	Displace Ca ²⁺ in body, rheumatism, mental fatigue, inactivation of essential enzymes, knotting of cartilages, keratinocytes, gastroenteritis, neuronal disorders [8, 251, 254, 284]
Copper (Cu)	oxidation states: 0, +1, +2,+3, most common/ toxic Cu ²⁺ [Cu(OH) ⁺ , Cu ₂ (OH) ₂ ²⁺], CuCO ₃ in aerobic and CuS in anaerobic environment, Cu ⁺ - a soft acid [248, 278, 286]	Mining, production of non-ferrous metals, iron and steel production, tanning, fertilizers, photovoltaic cells [8, 278]	1.3 [8, 31] 1.3 (USEPA) [248, 257] 2 (PUB) [255]	Damage to kidney, liver, inflammatory disorders, dyslexia, infected ears and hyperactivity in children, anemia, arthritis, allergies, cystic fibrosis [8, 31, 257]
Zinc (Zn)	oxidation states: 0, +2, most common Zn ²⁺ , bioavailable at high pH-hydrolyses around pH 7 – 7.5 into Zn ²⁺ [Zn(OH) ₂] [248, 278]	Volcanic eruption, coal and oil burning, iron and steel and non-ferrous metal production, soldering, pigments, cosmetics, phosphate fertilizer [8, 278]	5 [8], 3[282], 2 [249] 5 [252]	Carcinogenic, respiratory and neuronal disorders, Muscular degeneration, impotence, metal fume fever [8, 249]
Nickel (Ni)	oxidation states: -1to +4, most common Ni ²⁺ [278]	Volcanic eruption, coal and oil burning, production of alloys, phosphate fertilizer, Nickel-Chromium alloys, Nickel-Cadmium batteries,	0.02 [282] 0.03 [249] 0.07 (PUB) [255]	Carcinogenic – nasal, lung and laryngeal cancers, renal tumors, weight loss, dermatologic effects – hair loss, teratogenic effects – fused ribs, cleft

	Nuclear and aerospace applications, plumbing in marine environment, electroplating [249, 278]	palate [249, 278]

C-2 Diffusion co-efficient for various cations and anions

Table 6: Diffusion co-efficient for various cations and anions

Inorganic cations[265]		Inorganic anions	
Hydrated cation	D ($10^{-10} \text{ m}^2.\text{s}^{-1}$)	Hydrated anion	D ($10^{-10} \text{ m}^2.\text{s}^{-1}$)
Ag ⁺	16.48	OH ⁻	52.7
Al ³⁺	5.41	Br ⁻	20.8
Ba ²⁺	8.47	CO ₃ ²⁻	9.2
Be ²⁺	5.99	HCO ₃ ⁻	11.9
Ca ²⁺	7.92	Cl ⁻	20.3
Cd ²⁺	7.19	ClO ₄ ⁻	17.92
Co ²⁺	7.32	F ⁻	14.8
Cr ³⁺	5.95	I ⁻	20.45
Cs ³⁺	20.56	MnO ₄ ⁻	16.32
Cu ²⁺	7.14	NO ₃ ⁻	19.02
Fe ³⁺	6.04	PO ₄ ³⁻	6.1
Hg ²⁺	8.47	HPO ₄ ²⁻	7.59
K ⁺	19.57	H ₂ PO ₄ ⁻	9.59
Li ⁺	10.29	HS ⁻	17.3

Mg ²⁺	7.06	SO ₃ ²⁻	9.59
Mn ²⁺	7.12	HSO ₃ ⁻	13.31
Na ⁺	13.34	SO ₄ ²⁻	10.7
Ni ²⁺	7.05	HSO ₄ ⁻	13.3
Pb ²⁺	9.45	S ₂ O ₃ ²⁻	11.32
Zn ²⁺	7.03		

Appendix D

D-1 Classification of commonly used antibiotics

Table 7: Classification of antibiotics based on their characteristics

Class [268, 270]	Group [268]	Sub group [268]	Examples [268, 270]	Target cellular structure/ function [275]
β - lactams	Penicillins	BenzyI-penicillins	Phenoxypenicillin	Cell wall – block the supply of the enzyme transpeptidase [287]
		Aminopenicillins	Amoxicillin, ampicillin [288]	
		IsoxazolyIpenicillins	Oxacillin, cloxacillin, dicloxacillin [288]	
		Carboxypenicillins	Carbenicillin, ticarcillin	
		Acylaminopenicillins	piperacillin	
		Acylureidopenicillins [288]	Azlocillin, mezlocillin [288]	
	Cephalosporins	Cefalozin	Cefalozin	Cell wall - interrupt the formation of peptidoglycan layer of cell wall [289]
		Cefuroxim Cefotaxim Cefalexin	Cefuroxim Cefotaxim Cefprozil, Cefalexin	
	Carbpenems	-	Meropenem	
Tetracyclines	-	-	Doxycycline, Chlortetracycline, Tetracycline, Oxytetracycline	Protein synthesis – prevention of association between aminoacyl tRNA and 30 S ribosome [290]
Aminoglycosides	-	-	Gentamicin 1c, apramycin, neomycin, sesomycin, streptomycin	Protein synthesis – bind with 30 S ribosome to alter genetic code of protein [291]
Sulfonamides	-	-	Sulfamethoxazole, sulfanilamide, sulfadimethoxine,	Antimetabolite – prevention of formation of antimetabolites by

			sulfadimidine, sulfathiazole, sulfapyridine	competing for dihydropteroate synthetase [291]
Quinolones	-	-	Ciprofloxacin, cinoxacin, ofloxacin, enoxacin, lomefloxacin, norfloxacin, levofloxacin, sparfloxacin, gatifloxacin, moxifloxacin, trovafloxacin, nalidixic acid [292]	DNA – Conversion of DNA gyrase A and topoisomerase IV into toxic enzymes that breakdown the bacterial chromosome [293]
Macrolides	-	-	Erythromycin A, roxithromycin, spiramycin, clarithromycin, azithromycin, tylosin	Protein synthesis – bind to 50 S ribosome with a specific target unit from 23 S rRNA and some other ribosomal proteins [294]
Glycopeptides	-	-	vancomycin, teicoplanin [295]	Cell wall – bind to D- Ala-D-Ala ligase substrate and disrupt the cross linking of cell wall [296]