Electrical-phenotyping of the Bacterial Envelope Using Microfluidics

by

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Submitted to the Department of Mechanical Engineering in partial fulfillment of the requirements for the degree of Doctor of Philosophy at the

MASSACHUSETTS INSTITUTE OF TECHNOLOGY

September 2018

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Abstract  

The bacterial cell envelope is a complex multi-layered covering, crucial for cell viability and physiological capabilities. Phenotypic analysis of bacterial envelopes is challenging due to the small size and low cultivability of microbes. The emerging microfluidic techniques enable quantitative and nondestructive probing of cell envelopes by measuring their physical properties. This thesis demonstrates that phenotypic variations on bacterial envelopes change their surface polarizability—an intrinsic dielectric property—in a manner that can be distinguished by microfluidic dielectrophoresis (DEP). The three-dimensional insulator-based dielectrophoresis (3DiDEP), a microfluidic technique previously reported by our group, was optimized to explore the diverse surface phenotypes of bacterial electrochemical activity and lipopolysaccharide (LPS) biosynthesis. 

Electrochemically active bacteria transport electrons directly from their interior to external insoluble electron accepters, e.g. metal oxides or electrodes in electrochemical systems, via a process known as extracellular electron transfer (EET), holding an exciting promise in energy conversion and bioremediation. Using 3DiDEP, we demonstrate for the first time the strong correlation between microbial EET and cell surface polarizability, generalizable to three bacterial species with variant electrochemical activities, including Geobacter sulfurreducens, Shewanella oneidensis, and Escherichia coli heterologously expressing Shewanella EET pathways. We also applied 3DiDEP to achieve rapid quantification of LPS, the major component and virulence determinant in Gram-negative bacterial outer membrane. We examined E. coli mutant strains with various LPS components truncated, and show that structural diversity in LPS affects the trapping voltages required for 3DiDEP cell immobilization. Last but not least, we studied the interplay of electrothermal and induced charge electroosmosis (ICEO) flows, which can interfere DEP operations but are often overlooked in the design of iDEP systems. The effects of fluidic ionic strength, applied electric field, and insulating channel geometry on temperature rise and fluid velocities were investigated from a theoretical and experimental viewpoint. 

Taken together, this thesis introduces surface polarizability as a novel physical property for assessing microbial EET and LPS composition. Dielectrophoretic screening of bacterial envelope polarizability may unlock a vast repertoire of EET- and LPS-related biochemical applications, and will be useful as guidance for further DEP-based phenotypic analysis of a diverse array of cells and organisms.  

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Acknowledgments

I would like to thank my advisor, Professor Cullen R. Buie, for his consistent guidance and support throughout this work. I came to MIT with a background in mechanical design, but dived into the field of microfluidics and microbiology. I was attracted by the concept of developing biotechnologies to solve urgent biological questions. My advisor has always been encouraging me to broaden my knowledge in biology and establish collaborations with researchers in multidisciplinary backgrounds. He’s flexibility allows me to explore different biological questions and develop my enthusiasm and independence in research. He also has been supporting my career plan - to become a PI leading a research group in the future - and provided me with invaluable advice from his experience in the academia.

I would like to thank my committee members, Professor Scott R. Manalis and Professor Joel Voldman, for providing feedback and advice on all my research projects. They inspired me to enhance my research with a more rigorous experimental design, and to extend my research into a broader application generalizable to multiple bacterial species. They also shared with me valuable experiences for my professional development.

Also, my accomplishments in the PhD program would not have been possible without the help from my colleagues in the Laboratory for Energy and Microsystems Innovation. I would like to thank Dr. Will Braff for training me on fabricating and operating microfluidic devices; Dr. Andrew Jones III for helping me to deal with the slow-growing G. sulfurreducens and discussing the experimental design problems; Dr. Zhifei Ge for guiding me through the bench-top operations when I first joined the lab; Dr. Alisha Schor for teaching me how to bond and align multiple PDMS layers; Dr. Naga Dingari for collaborating with me on modeling the nonlinear electrokinetic effects to solve the problem that I occasionally ran into, which eventually becomes a paper published; Dr. Chelsea Catania and Dr. Chris Vaiana for always keeping their door open for discussion; as well as Dr. Paulo Garcia, Dr. Youngsoo Young, Dr. Pei Zhang, Dr. Jeff Moran, Kameron Conforti, Laura Gilson, Rameech McCormack, Sijie Chen, Hyungseok Kim, and our administrator Maral Banosian.

I would like to express my appreciation to Professor Derek Lovley, Professor Jeffery Gralnick, and Dr. Caroline Ajo-Franklin for kindly providing bacterial strains for my research project, and giving me feedback on my study of electrochemically active bacteria. I acknowledge Professor Christopher Hayes and Tiffany Halvorsen for providing the E. coli LPS mutants.

Last but not least, I would like to thank my mother, Yanqin Tang, and my father, Dr. Yuxing Wang, who have always been supportive throughout these years. My enthusiasm toward research was initially triggered by them, who are both professors in China. Their persistent love, encouragement and understanding give me strength and confidence to face difficulties, and to keep an optimistic mindset to move forward.
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Chapter 1

Introduction

1.1 Cell Envelope Studies Uncover Microbial Enigmas

The bacterial cell envelope, a chemically complex multi-layered covering that lies outside of the cytoplasm, is crucial for cell viability and physiological capabilities. [1] Although we should never judge a book by its cover, cell envelope studies provide us with a wealth of information to understand microbial behaviors, such as pathogenicity [2], biofilm formation [3, 4], antibiotic resistance [5], and electron transfer [6].

Bacterial cell envelopes can be classified into two major groups (Figure 1.1). Moving outwards, Gram-positive bacteria are surrounded by a cytoplasmic cell membrane, which itself is surrounded by a thick cell wall consisting of a cross-linked peptidoglycan (PG) network. [1] Threading through these PG layers are long anionic polymers including teichoic acid and lipoteichoic acid. In contrast, Gram-negative cell envelopes are generally composed of three layers: the cytoplasmic membrane, a cell wall much thinner than is found in Gram-positives, and an outer membrane (OM). The OM
is the outermost layer and a unique feature of Gram-negative bacteria. It is an asymmetric lipid bilayer, whose inner leaflet is composed of phospholipides, whereas the outer leaflet is principally composed of lipopolisacchoride (LPS). [1] Additionally, the OM recruits a number of proteins, including transmembrane proteins (e.g. prorin proteins), which serve as membrane channels, and lipoproteins, which are embeded in the inner leaflet of the OM and show a broad-ranging functionality. Each building block of the cell envelope performs distinct or overlapped functions, and they collaboratively act as an adaptive barrier playing multiple indispensable roles, such as protecting the organism from the changing and often hostile environment, regulating transport, providing sites for energy reactions and synthesis, as well as facilitating colonization and intercellular communications. The following sections summarize three important physiological properties shown to be strongly associated with bacterial envelope properties.

1.1.1 Cell viability and antibiotic susceptibility
A surge of studies has shown that cell envelope phenotyping can provide insights into the complex bacterial physiology. For instance, cell envelope integrity and permeability can be strongly associated with bacterial viability and antibiotic susceptibility. [7-10] First, the synthesis of PG cell wall is a target of many commonly used antibiotics, such as beta-lactams (including penicillins and cephalosporins) and glycopeptide antibiotics (e.g. vancomycin). [11] Beta-lactams block the cross-linking between the PG monomers through inactivation of penicillin-binding proteins (PBP), which catalyze peptide bond formation. [12] Glycopeptide antibiotics inhibit PG cross-linking through binding with PG unites directly to block transglycosylase and transpeptidase activity. [13] Other cell-wall-targeting drugs can inhibit PG synthesis by blocking the transport of PG monomers across the cytoplasmic membrane (e.g. Bacitracin). [14] Successful treatment with these antibiotics results in compromised cell wall and osmotic bacterial lysis. Second, for antibiotics targeting intracellular components, such as protein synthesis (e.g. streptomycin and tetracycline) and nucleic acids (e.g. fluoroquinolones), the cell envelope serves as a permeability barrier. Therefore, some species of bacteria with low-permeability membrane barriers are intrinsically resistant to many antibiotics. [10] Mycobacterium tuberculosis, the causative agent of Tuberculosis
leading to 1.7 million deaths worldwide in 2016 (reported by CDC\textsuperscript{1}), produces unusual cell wall structure, 60\% (by weight) of which is occupied by lipids that consist mainly of long-chain fatty acids. [15] This unique cell wall structure renders \textit{M. tuberculosis} resistance to multiple antibiotics. Additionally, membrane-associated efflux pumps actively regulate the intracellular penetration of antibiotics, and constitute an important class of resistance. [16] Last but not least, bacteria are able to acquire antibiotic resistance via dynamic modification of their cell envelopes. For example, \textit{Staphylococcus aureus} was shown to acquire resistance to vancomycin by substituting the D-alanyl-D-alanine residues (to which vancomycin can bind) in the PG chain with D-alanyl-D-lactate, a dipeptide with substantially lower affinity for the antibiotic. [17] Moreover, several Gram-negative bacterial species, such as \textit{Pseudomonas aeruginosa}, \textit{Acinetobacter baumannii}, and \textit{Escherichia coli}, have shown resistance to polymyxin due to modifications of lipid A phosphates in their LPS with positively charged groups. [18-21] Such lipid A modification reduces the net negative charge in the outer membrane, shading the cell from electrostatic attraction by the positively charged antibiotic molecule.

\textbf{1.1.2 Pathogenicity}

Cell envelope studies also shed light on the mechanisms of bacterial pathogenicity. For example, bacterial LPS (also termed endotoxin), the major outer membrane component in Gram-negative bacteria (Figure 1.1), is a representative virulence determinant that allows mammalian cells to recognize pathogen invasion and triggers innate immune responses. [22] LPS is a negatively charged molecule consisting of a preserved lipophilic component, lipid A, which anchors to the outer membrane, and polysaccharides, which is diverse in length and composition amongst the different Gram-negative bacterial species. [23] A more detailed discussion on LPS chemical structure and physiological roles can be found in Chapter 4.1. LPS released from invading pathogens is recognized by the innate immune system through the pattern recognition receptors, such as the Toll-like receptor 4 (TLR4)/MD-2 complex, which initiates production of cytokine that is crucial for clearance of bacterial infection. [23] However, a variety of human pathogens are able to escape from host immune responses by modifying their LPS structures. Pathogens, including

\textsuperscript{1} Data provided by Centers for Disease Control and Prevention (CDC), https://www.cdc.gov/tb/statistics/default.htm
Yersinia pestis and Francisella tularensis, have been shown to change their lipid A structures into a hypo-acylated form with weakened activity for TLR4 signaling, and thereby facilitate bacterial evasion. [22] Additionally, LPS in several pathogens contain O-antigen structures that closely resemble human glycosphingolipids, which has been shown to play a role in bacterial “camouflage”. [22, 24] For instance, Helicobacter pylori, a prevalent gastroduodenal pathogen of humans, produces LPS O-antigen units exhibiting mimicry of Lewis antigens, carbohydrates that are also expressed by the gastric epithelium in humans, and has been suggested to potentiate gastric colonization, adhesion and immune evasion. [22, 24]

1.1.3 Electron transport and membrane potential
In addition to acting as a protective barrier to block toxic agents and a sensor to interact with the environment, bacterial cell envelopes play key roles to regulate cross-membrane transport of nutrients, electrons, and ions, etc., which is strongly associated with microbial metabolism. For example, bacteria enable electron transport across the cell envelope via a series of electron carriers, during which energy can be released and conserved as a proton motive force to create adenosine diphosphate (ATP), or to drive other cellular tasks, such as secondary active solute transport, protein secretion, and cell motility. [25, 26] This electron transport process in cell membranes is crucial to empower cell growth, and occurs in a wide range of scenarios including membranes of prokaryotes, mitochondria, and chloroplast. [25] The continuous electron flow across cell membranes is regulated by a number of membrane associated electron carrier proteins (e.g. cytochromes) and lipids (e.g. quinones), and is coupled with the extracellular translocation of protons (H+), establishing a membrane potential, outside positive. Once established, the membrane potential can energize the secondary flow of other ions, such as the influx of potassium ions. [25] Studies on the electron transport in bacterial cell envelope hasten the development of microbial electrochemical techniques. For example, a class of bacteria, such as the dissimilatory metal reducing bacteria (DMRB), are capable of anaerobically respiring on insoluble minerals and metals by directly transporting electrons from their interior towards these extracellular electron acceptors via a process known as extracellular electron transfer (EET). [6] The EET process has gained intensive attention due to its potential in electrochemical systems (e.g. microbial fuel cells) [27] and bioremediation [28]. The EET process will be further discussed in Chapter 3.1. Additionally,
studying the dynamics of membrane potential in response to different external cues (e.g. electrical pulses and chemical gradients) has become one of the most exciting topics in biophysical and biotechnological areas, including electroporation and electrotaxis. [29-31] For instance, during electroporation, cells are exposed to an external electric field pulse, which induces a transient local transmembrane voltage superimposed onto the resting membrane potential. Once the transmembrane potential exceeds a threshold value, pores are formed in the cell membrane to allow intracellular penetration of foreign molecules including nucleic acids, plasmids, and peptides. [29, 30] However, a detailed mechanistic understanding in transmembrane potential and pore dynamics remains exclusive. [30] Moreover, recent studies show that dynamics in membrane potentials and ion transport also play significant roles in cell signaling and electrical communications within bacterial communities. Süel and coworkers showed that in bacterial biofilm communities, spatially propagating waves of potassium can induce synchronized waves of membrane potentials (via the potassium ion-channel), which coordinates metabolic states among cells in the interior and periphery of the biofilm, and allows long-range electrical communication to cooperatively combat with nutrient limitations. [32, 33]

1.2 Challenges in Bacterial Cell Envelope Studies

Phenotypic analysis of bacterial cell envelope is a challenging task, due to the small size (ca. 1 μm) [25], high biological diversity, and low cultivability [34] for the majority of microorganisms existing in the universe. A variety of phenotyping techniques have been established, providing valuable information for bacterial cell envelope studies, but each technique has unique advantages and limitations. Culture-based phenotyping is the current gold standard phenotyping method in many circumstances, such as the measurement of cell fitness and antibiotic tolerance, but is normally inapplicable to slow-growing or uncultured microbes. For instance, antimicrobial susceptibility testing (AST) is one of the most important tasks in clinical microbiology laboratories in order to detect possible drug resistance in pathogens and to assure the best drugs of choice for particular infections. The most widely used AST method relies on culture of target pathogen on solid growth substrates (e.g. agar plates) exposed to a varying concentration of antibiotics, but this approach often involves tedious dilution steps and time-consuming incubation process (counted
by days), and thus is too slow to be implemented at the point of care. [35] Genomic methods, such as polymerase chain reaction (PCR) and RNA sequencing, detect ‘molecular phenotype’ by quantifying preselected genes modulated specifically by the phenotype of interest, eliminating the cultivation step. However, this molecular phenotyping is not generalizable to different bacterial species, and requires prior knowledge in genotype-to-phenotype relationship. [36] Additionally, the accuracy of this approach relies on precise thermal cycle control and trained laboratory personnel. Affinity-based phenotypic measurements, such as western blot and enzyme-linked immunosorbent assay (ELISA), provides high specificity and high sensitivity using the antigen-antibody reactions. However, these methods are only applicable when the specific affinity reagents are available. [37] Mass spectrometry (MS) methods enables accurate determination of the structure and composition of target chemical compounds and biological molecules according to their mass-to-charge ratio. However, similar to the affinity-based approach, the MS methods require preparation steps including cell lysis, protein extraction, fragmentation, and purification, which often involves toxic chemicals and requires a specific skillset. [37, 38]

It turns out that bacterial phenotypic studies have such complexity that no single technique is adequate for establishing a complete understanding. The aforementioned techniques have their specific limitations, and thus are often used in combination. Meanwhile, complementary innovative methods, including microfluidic technologies, is advancing quickly, and may unlock a vast repertoire of phenotyping strategies.

1.3 Microfluidic Systems for Cell Envelope Phenotyping

It has been shown that many complex physiological variations in cell envelopes can be detected quantitatively and nondestructively by measuring physical properties of the cell envelope. Quantification of cell biophysical properties is hastened by microfluidic technologies, which outstrip other methods with a number of advantages, including high throughput and low reagent cost. For example, the mechanical properties of bacterial cell envelope are strongly associated with species, cell survival and division. [39] Vadillo-Rodriguez et al. [40] utilized atomic force microscopy (AFM)-based approach to quantify the viscoelastic properties of bacterial envelope at
a single-cell level, and observed distinct creeping behaviors between Gram-positive and Gram-negative bacteria. Wang et al. [41] used an optical trap to measure the bending rigidity of live *E. coli* and illustrated the contribution of actin-homolog MreB to bacterial cell stiffness. These approaches allow high accuracy single-cell measurements, but are normally labor-intensive. Tuson et al. [42] measured bacterial envelope stiffness using hydrogel encapsulation, but their method requires permanent cell immobilization. Microfluidic devices can be adapted to surmount these drawbacks. Sun et al. [43] developed a microfluidic platform to profile the stiffness of individual bacteria, with 100-folded throughput compared to the mentioned approaches, minimal sample preparation, and no chemical immobilization or labeling. However, high-throughput cell envelope phenotyping for bacteria is still a bottleneck compared to the cell envelope analysis for eukaryotic cells. Gossett et al. [44] developed an automated microfluidic system capable of using hydrodynamic stretching to quantify single-cell deformability at 2,000 cells/s. Gossett’s work also linked cell deformability to leukocyte malignancy in pleural fluids and pluripotency of embryonic stem cells, enabling applications in clinical diagnostics and stem cell characterization. However, the hydrodynamic stretching method is not directly applicable to bacteria, which are typically 10 times smaller than mammalian cells.

As a second example, electrical properties of cell envelopes also provide insight for understanding the complex physiological states in the cell. The patch-clamp technique, introduced by Neher and Sakmann in 1976 [45], is the conventional research tool to measure ion channel current and transmembrane potential across the cell membrane. Cell membrane is aspirated into a (conductive fluid-filled) glass micropipette, forming a resistance seal, and thereby ion current flowing through the pipette (containing an electrode) can be measured via an amplifier. Lau et al. [46] adopted this concept and built a microfluidic patch-clamp array to characterize seal resistances of CHO cells. However, the patch-clamp technique, used to probe eukaryotic cells, involves mechanical cell trap, and thus is not readily applicable to bacteria. Moreover, cell dielectric properties can be determined using electrorotation (ROT) [47-53] or impedance measurements [54-57]. The ROT approach [47-53] involves an AC rotational electric field created by quadrupole electrodes, each one connecting to an AC signal with a 90° phase difference from each other. A cell placed at the center of the quadrupole electrodes forms an electrical dipole moment that rotates in synchronism with the
external electric field but lags behind by a phase factor associated with the dielectric properties of the cell and surrounding media, as well as the electric field frequency. Cell rotational direction/speed in response to a spectrum of applied frequency (typically ranging from 1 kHz to 1 GHz) is measured to extract cell dielectric properties, such as specific membrane capacitance and cytoplasm conductivity. [47-53] So far, the ROT method is limited to study either large cells, such as lymphocytes [58], erythrocytes [58], leukocytes [48, 52], yeast cells [50], and intestinal parasites [47], or large microbeads (6 µm) coated with bacteria [49, 53], with only one exception [51], where ROT was applied to characterize individual living and heat-treated E. coli cells. In addition to ROT, microfluidic-based impedance measurement offers another noninvasive method for cell electrical phenotyping. [54-57] Impedance-based methods often involve detection areas where parallel or coplanar electrode pairs are embedded on the microchannel wall, connected to AC voltage signals. [55, 56] Cells trapped in (or flowing through) the detection area changes the impedance value within the channel, and the impedance measured over a wide frequency range (or at a prescribed frequency) permit dielectric characterization of different compartments of the cell. Several impedance-based methods have been applied to study bacteria. For example, Bernabini et al. [54] developed a microfluidic impedance flow cytometer, which combines hydrodynamic focusing and impedance characterization, to distinguish E. coli from 1 µm polystyrene beads. Haandbæk et al. [57] demonstrated a resonance-enhanced impedance cytometer, which incorporates a discrete inducer in series with the measurement electrodes in the microfluidic channel, to achieve discrimination of E. coli and Bacillus subtilis based on dielectric properties. However, high-sensitivity impedance measurement of bacteria is still challenging, partially because the non-uniform electric field generated by the planar microelectrodes can result in heterogeneous impedance signal for identical cells at different positions when passing through the detection electrodes.

Dielectrophoresis (DEP), first adopted by Pohl [59], is another technique that has been widely integrated into microfluidic systems to concentrate [60, 61], sort [62-64], and characterize [65-67] sub-micron bioparticles (e.g. bacterium and virus) based on their dielectric properties. During DEP, a polarizable particle (not necessarily charged) placed in a non-uniform electric field experiences an induced dipole moment, which interacts with the non-uniform electric field resulting in a net
force that either drives the particle towards (positive DEP) or away from (negative DEP) the strong electric field region, depending on the relative polarizability of the particle and the surrounding medium. [59] Since the DEP force exerted on the particle is proportional to the gradient of electric field squared, $\nabla \left( E^2 \right)$, a proper design of the electric field distribution is crucial to achieve high-sensitivity in DEP operations. Unlike the mentioned ROT and cell impedance measurements that utilized high frequency electric fields to detect cell internal properties, DEP induced by DC electric fields enables evaluation of cell surface properties exclusively [62, 65-68] (see a detailed explanation in Chapter 3.5). The electric fields are mainly established either by fabricating metal electrodes directly into microfluidic channels [48, 63, 65, 67, 69], or by controlling the insulating channel geometries and passing a remotely applied potential drop across the microchannel [60, 61, 64, 66, 68]. The later one, referred to as insulator-based dielectrophoresis (iDEP), outperforms the embedded electrode systems by diminishing a number of issues, such as Joule heating, electrode fouling, fabrication complexities due to metal deposition in the microchambers, and electrochemical reactions on the electrode surface, which allows the use of DC signals. [70] Previous work by my colleague, Braff et al., has shown that three-dimensional insulator-based dielectrophoresis (3DiDEP) provides a high sensitivity approach to distinguish bacteria with subspecies level resolution, [66, 68] suggesting an exciting promise to apply the 3DiDEP technique towards broader cell envelope phenotyping applications. However, this approach, and many other iDEP-based bacterial cell characterization studies, relies on measuring the DEP force exerted on the cell instead of measuring cell dielectric properties. The DEP force is contingent on both cell dielectric properties and cell morphology (see Chapter 2.1). Decoupling the effect of cell morphology and size is important for accurate electrical phenotyping of the bacterial envelope in iDEP systems.

### 1.4 Study Objectives and Thesis Organization

In this thesis, the previously reported 3DiDEP technique [66, 68] was advanced by combining the 3DiDEP trapping with both linear electrokinetic and cell morphology measurements to extract an intrinsic cell envelope dielectric property – cell surface polarizability. The new three-step analyzing platform introduces cell surface polarizability as a novel parameter to achieve
quantitative electrical phenotyping of various bacterial envelope features, including cross-
membrane electron transport and LPS compositions. The hypothesis is that phenotypic variations
on bacterial envelopes measurably influence cell surface polarizability, which can be detected
using microfluidic DEP. A variety of bacterial species and mutant strains were used to examine
the feasibility and generalizability of the 3DiDEP-based electrical phenotyping platform.
Additionally, potential confounding factors influencing the detection sensitivity in iDEP systems
were analyzed from a theoretical and experimental viewpoint.

Chapter 2 illustrates the working principle and underlying physics of the 3DiDEP platform.
Mathematical derivations are demonstrated for the first time to extract bacterial surface
polarizability values from 3DiDEP experiments, and more importantly to capture cell
morphological effects on DEP-based analysis, which are often overlooked in previous studies.

Chapter 3 demonstrates the application of the 3DiDEP platform towards quantitative electrical
phenotyping of the extracellular electron transfer (EET) occurring in the cell envelopes of
electrochemically active bacteria. We show for the first time that microbial EET (a cellular
physiological property) is correlated with cell surface polarizability (an electrical property) that
can be easily measured by microfluidic DEP. Surface polarizability was measured for two different
species of dissimilatory metal-reducing bacteria (DMRB), *Geobacter sulfurreducens* and
*Shewanella oneidensis*, as well as *E. coli* heterologously expressing *Shewanella* EET conduits,
confirming the generalizability of our hypothesized correlation between microbial EET and cell
surface polarizability.

Chapter 4 demonstrates the 3DiDEP-based electrical phenotyping of another bacterial envelope,
LPS, which is the major component and virulence determinant in Gram-negative bacteria. *E. coli*
mutant strains with various LPS compositions were examined using the 3DiDEP device, showing
for the first time an almost linear correlation between LPS phenotype and the trapping voltage
required for 3DiDEP cell immobilization.
Chapter 5 presents a theoretical and experimental study on the interplay of electrothermal and induced charge electroosmosis (ICEO) flows near microchannel constrictions, which are potential confounding factors that interfere DEP operations and are often overlooked in the design of iDEP systems. The effects of fluidic ionic strength, applied electric field, and insulating channel geometry (which are crucial design factors for iDEP systems) on temperature rise and fluid velocities were studied experimentally with supporting analytical estimations and numerical simulations. Approximate analytical expressions for electrothermal and ICEO velocity scales induced by a wide range of experimental conditions are provided as a quick guidance for the design of electrokinetic systems.

Finally, Chapter 6 discusses the major conclusions and a broader impact of this study, as well as opportunities for further improvement and future work.
Chapter 2

Assessment of Bacterial Surface Polarizability Using Three-dimensional Insulator-based Dielectrophoresis (3DiDEP)

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2.1 Mechanism of 3DiDEP cell trapping

Polarizability represents the tendency to form electric dipoles in a material (not necessarily charged) subjected to externally applied electric fields. Cell surface polarizability represents the overall dielectric properties at the cell/media interface. It should be noted that we consider polarizability as a physical property adopted from the area of electromagnetics, rather than the biological concept (e.g. cell polarity) defined as the ability to form asymmetric organization of cellular components and shape as in the case of cell division and cell migration.

Cell surface polarizability was quantified by the Clausius-Mossotti factor ($\kappa_{CM}$) [59], a measure of the relative polarizability of the cell compared to the surrounding media. In order to measure $\kappa_{CM}$, DEP-based cell trapping was performed using the 3DiDEP microchannels (Figure 2.1a) employing a linear sweep analysis, in which a DC voltage drop applied across the microchannel increases linearly with time (Figure 2.1b). [66] The microchannel contains a 3D insulating constriction with a cross-sectional area 100 times smaller than that of the main channel, creating a strong electric field gradient in the vicinity of the constriction (Figure 2.1c and d). In the opening regions of the microchannel, bacteria are driven towards the constriction by linear electrokinetics.
Figure 2.1 **Working principle of the 3DiDEP microfluidic device.** a) A 3DiDEP microfluidic device with an array of multiple microchannels. b) A DC potential difference increasing linearly with time at 1 V/s was applied across the channel. c) A magnified view of the microchannel highlighting the constricted area. d) A schematic depicting the 3DiDEP trapping principle.
(EK). The resulting electrokinetic velocity comprises the effects of both electroosmosis and electrophoresis, and is proportional to the applied electric field ($\vec{E}$) as

$$\vec{U}_{EK} = \mu_{EK} \vec{E}$$

(2-1)

where $\mu_{EK}$ is the combined linear electrokinetic mobility [64]. Bacterial cells investigated in this study are rod-shaped, and thus can be modeled as ellipsoidal particles with semi-axes $a > b = c$. [63] Since both shear and electro-orientation tend to align the prolate cell in the direction of flow (i.e. the electric field direction), the Stokes’ drag exerted on the bacterium is estimated as

$$\vec{F}_{\text{drag}} = 6\pi \xi \eta a \left( \vec{U}_{EK} - \vec{U}_p \right)$$

(2-2)

where $\eta$ is the viscosity of the surrounding medium and $\vec{U}_p$ is the particle velocity. The Perrin friction factor [71] $\xi$ is

$$\xi = \frac{\sqrt{1 - p^2}}{\ln \left( 1 + \sqrt{1 - p^2} \right)}$$

(2-3)

with $p = b/a$. The Perrin friction factor represents a multiplicative adjustment to the drag force of a rigid spheroid, relative to the corresponding friction exerted on a spherical particle of the same volume. For spherical cells (e.g. a coccus), $\xi = 1$. Near the constricted region, the rod-shaped bacterium experiences a DEP force induced by the DC electric field [72],

$$\vec{F}_{\text{DEP}} = 2\pi ab^2 \varepsilon_m \kappa CM \nabla \vec{E}^2$$

(2-4)

where $\varepsilon_m$ is the permittivity of the surrounding medium. As shown in Figure 2.1c, the bacterium is immobilized when the DEP force balances the driving force due to linear electrokinetics along the electric field direction. [60, 64, 68] This criterion for 3DiDEP immobilization of a single cell can then be expressed as

$$6\pi \xi \eta a (\vec{U}_{EK} - \vec{U}_p) \cdot \vec{E} + 2\pi ab^2 \varepsilon_m \kappa CM \nabla \vec{E}^2 \cdot \vec{E} = 0$$

(2-5)

Substituting Equation (2-1) and $U_p = 0$ gives
\[ \mu_{\text{EK}} \vec{E} \cdot \vec{E} + \mu_{\text{DEP}} \left( \nabla \vec{E}^2 \right) \cdot \vec{E} = 0 \]  

(2-6)

where the DEP mobility, \( \mu_{\text{DEP}} \), is specified as

\[ \mu_{\text{DEP}} = \frac{b^2 \varepsilon_m \kappa_{\text{CM}}}{3 \eta \xi} \]  

(2-7)

When an increasing DC voltage drop (Figure 2.1b) is applied, bacteria in the microchannel start with passing through the 3DiDEP constriction until a threshold electric field is reached such that Equation (2-6) holds. Once the 3DiDEP immobilization is initiated, with the applied voltage further increasing, cells began to accumulate at the edge of the microchannel constriction.

### 2.2 Workflow for the quantification of cell surface polarizability

As shown in Figure 2.2, the cell surface polarizability is determined by three parameters measured separately, namely, (1) The minimum applied voltage (“trapping voltage”) required for the onset of 3DiDEP immobilization, (2) the linear electrokinetic mobility (\( \mu_{\text{EK}} \)), and (3) the bacterial morphology, which defines \( b^2 / \xi \).

#### 2.2.1 Determine the critical electric field using 3DiDEP

Assuming the electrothermal and other nonlinear electrokinetic effects are negligible (see Chapter 5), the electric field distribution within the microchannel is solely determined by the trapping voltage. The trapping voltage is measured using the 3DiDEP devices via the following procedure.

1. **Fabrication and preparation of the microfluidic 3DiDEP device**

   The 3DiDEP device was fabricated by CNC micromachining a piece of poly (methyl methacrylate) (PMMA) sheet and bonding it with another blank PMMA chip using a solvent-assisted thermal binding process after cleaned both chips with acetone, methanol, isopropanol, and deionized (DI) water in sequence. \[73\] Fluid reservoirs were then attached on top of the chips using a two-part
Figure 2.2 A workflow for the quantification of cell surface polarizability. STEP I, the trapping voltage required for the onset of cell immobilization was determined using 3DiDEP, and inputted as boundary conditions into a COMSOL 3D model to estimate the critical electric field. STEP II, particle image velocimetry (PIV) was utilized to monitor cell velocity changes in response to an increasing external electric field to determine the linear electrokinetic mobility. STEP III, cell morphology was imaged and quantified using an ellipsoidal fit to extract the values of cell semi-axes and the corresponding Perrin coefficient. Cell surface polarizability is derived from these three parameters according to Equation (2-6) and (2-7).
epoxy (Figure 2.1a). The channels are 1 cm in length, including a 50 µm long 3D constricted region with a cross-sectional area of 50 × 50 µm² in the center. The constriction bridges the two main channels where the cross-sectional area is 500 × 500 µm², yielding a constriction ratio of 100. The high 3D constriction ratio enables high sensitivity for 3DiDEP characterization at low applied voltages. More detailed description of the fabrication methods and channel geometry can be found elsewhere. [66, 68] It is essential to maintain a consistent surface charge on the PMMA channel walls to generate constant electroosmotic flows. As a result, a conditioning process was performed before each 3DiDEP and linear electrokinetic mobility measurement. Each microchannel was flushed with 100 µM potassium hydroxide, DI water, and the DEP buffer solution sequentially at 500 µL/min for ten minutes. The straight PMMA microchannels used for linear electrokinetic mobility measurements were 1 cm × 500 µm × 50 µm (length × width × depth), fabricated and primed using the same fabrication technique and conditioning process described above.

II. Sample preparation for 3DiDEP
Bacterial cells reaching stationary phase were fluorescently labeled using 5 - 20 µM of SYTO® BC Green Fluorescent Nucleic Acid Stain (ThermoFisher Scientific) in their native media, then centrifuged at 4,000 rpm for 4 min. The cells were rinsed once and well mixed using a vortex mixer in their native growth media to remove the dye before being centrifuged again, and then resuspended in the DEP buffer. The DEP buffer solution (final pH = 6.8) was prepared by adding DI water to 1X Phosphate Saline Buffer Solution (PBS) until the solution conductivity was nearly 100 µS/cm. The DEP buffer also contains 1-2% v/v glycerol for an osmolality matching that of the growth medium.

III. Determine the trapping voltage using 3DiDEP
The 3DiDEP microchannels were observed carefully via microscope to ensure that bubbles were removed if presented in the channels. Excessive liquid was removed from the fluidic reservoirs. The cell suspension in the DEP buffer was diluted to an OD600 ca. 0.05, and a volume of 140 µL was introduced into the 3DiDEP microchannel via the fluidic reservoirs. Pressure difference across the microchannel was eliminated before each experiment. A ‘linear sweep’ DC voltage difference increasing linearly with time at 1 V/s from 0 V to 100 V was applied across the channel via an
HVS-448 high voltage power supply (LabSmith) controlled by a customized LabVIEW program. The SYTO BC fluorescence intensity increased with time as bacterial cells accumulated near the constricted region, and was recorded by time lapse image sequences captured at 1 fps using a CoolSNAP HQ2 cooled CCD camera (Photometrics) fitted to an inverted fluorescence microscope (Nikon). The fluorescent intensity data (arbitrary unit, background subtracted) near the constriction was averaged over the region of interest (yellow dashed box in Figure 2.3) using ImageJ, and plotted against time (i.e. the applied voltage). Then, the fluorescent intensity versus applied voltage was fitted into a polyline with two segments, whose intersection point was taken as a variable optimized using the least squares method by a customized MATLAB R2015b (MathWorks) code. If we denote the observed N pairs of (applied voltage, fluorescent intensity) data as \( \{(x_i, y_i), i = 1,2,...,N\} \), the fitting curve can be expressed as

\[
f(x) = \begin{cases} 
  a_1x + b_1, & x \leq x_n \\
  a_2x + b_2, & x > x_n 
\end{cases}
\] (2-8)

where the data space of applied voltage, \( X = \{x_1 < x_2 < ... < x_N\} \), is separated into two domains, \( X_1 = \{x_1 < x_2 < ... < x_n\} \) and \( X_2 = \{x_{n+1} < x_{n+2} < ... < x_N\} \). According to the least-squares approach, the goal is to find the \( n \) from \( \{4,5,...,N-4\} \) and the corresponding fitting parameters \( (a_1, a_2, b_1 \text{ and } b_2) \) such that the sum of squared residuals is minimized. Linear regression gives

\[
a_1 = \frac{n \sum_{i=1}^{n} x_i y_i - \sum_{i=1}^{n} x_i \sum_{i=1}^{n} y_i}{n \sum_{i=1}^{n} x_i^2 - \left( \sum_{i=1}^{n} x_i \right)^2}, \quad b_1 = \frac{\sum_{i=1}^{n} y_i - a_1 \sum_{i=1}^{n} x_i}{n} \] (2-9)

\[
a_2 = \frac{(N-n) \sum_{i=n}^{N} x_i y_i - \sum_{i=n}^{N} x_i \sum_{i=n}^{N} y_i}{(N-n) \sum_{i=n}^{N} x_i^2 - \left( \sum_{i=n}^{N} x_i \right)^2}, \quad b_2 = \frac{\sum_{i=n}^{N} y_i - a_2 \sum_{i=n}^{N} x_i}{N-n} \] (2-10)
Figure 2.3 A representative plot showing the 3DiDEP image analysis to extract the trapping voltage. The measured fluorescent intensity data (circles) is plotted against the applied voltage. The red and blue lines are fitting curves, whose intersection defines the trapping voltage. Inserted are microscopic images (background subtracted) taken before, at the onset of and after cell immobilization. The yellow dashed box indicates the region of interest. Scale bar: 100 μm.
In other words, the minimization problem is to find \( \min_{n,a_1,b_1,a_2,b_2} S_e(n,a_1,b_1,a_2,b_2) \), for
\[
S_e(n,a_1,b_1,a_2,b_2) = \sum_{i=1}^{n} \left[ y_i - (a_1 x_i + b_1) \right]^2 + \sum_{i=n}^{N} \left[ y_i - (a_2 x_i + b_2) \right]^2
\]
(2-11)
The applied voltage corresponding to the determined intersection point of the two segments, \( V_{trap} = \frac{b_2 - b_1}{a_1 - a_2} \), was extracted as the trapping voltage (Figure 2.3).

IV. Determine the critical electric field using COMSOL simulation
A 3D numerical simulation was carried out using COMSOL 5.1 Multiphysics Software (COMSOL) to evaluate the local electric field intensity and gradient. The electric potential boundary condition at one end of the microchannel was prescribed as the measured trapping voltage, while the other end was set to be grounded (Figure 2.4a). Figure 2.4c-e show the estimated nondimensional electric field \( \tilde{E} \) normalized by \( V_{trap} / l_c \), where \( l_c \) is the length of the constriction (50 \( \mu \)m), and the nondimensional group, \( \tilde{\beta} = \frac{\nabla \tilde{E}^2 \cdot \tilde{E}}{\tilde{E} \cdot \tilde{E}} \cdot \frac{l_c^2 \cdot V_{trap}}{V_{trap}} \). \( \tilde{\beta} \) indicates the ratio between the linear electrokinetic mobility and the DEP mobility (Equation 2-6). In the numerical model, edges at the insulating constriction were rounded with a fillet radius of 0.5 \( \mu \)m to reduce the singularity effects (Figure 2.4d). No significant difference in the simulation results was observed if using a larger fillet radius. Values of \( \frac{\nabla \tilde{E}^2 \cdot \tilde{E}}{\tilde{E} \cdot \tilde{E}} \) was averaged over a line 4 \( \mu \)m away from the constriction edge (Figure 2.4d and 2.4e), to ensure a good estimation of the critical electric field at the location of cell immobilization (Figure 2.3), as well as eliminating significant singularity effects.

2.2.2 Determine the linear electrokinetic mobility
The combined linear electrokinetic mobility (\( \mu_{EK} \)) of each bacterial strain was determined by Particle Image Velocimetry (PIV) using a PIVlab MATLAB program. [74] The PIV measurement will be discussed with more details in Chapter 5. Bacterial motion was tracked in a straight PMMA microfluidic channel with the ‘linear sweep’ DC voltage applied. Time lapse image sequences
Figure 2.4 **A 3D numerical model to estimate the critical electric field for 3DiDEP.** a) The electrical potential distribution across the microchannel when the trapping voltage is taken as the prescribed boundary condition. The magnified image shows the magnitude distribution of the electric field near the microchannel constriction. b) A table of parameters and material properties for the numerical simulation. c) XY-distribution of the electric field magnitude near the insulating constriction. d) XZ-distribution of the electric field magnitude (left) and $\tilde{\beta}$ (right) near the insulating constriction. Edges of the constriction were rounded with a fillet radius of 0.5 $\mu$m to reduce singularity effects. e) XY-distribution of $\tilde{\beta}$ near the insulating constriction. Red crosses in (d) and red dashed lines in (e) indicate the position to take line average.
were recorded using a CCD camera, and the PIV program was used to yield the velocity field (Figure 2.2) in the straight microfluidic channel in response to the varying applied voltage. The average velocity versus applied electric field was fitted linearly with the least-squares method, and the best-fit slope was taken as the linear electrokinetic mobility for each examined bacterial strain.

Along with the numerically estimated data of \( \left( \nabla E^2 \right) \cdot \vec{E} \), DEP mobility (\( \mu_{\text{DEP}} \)) of bacterial strain studied can be extracted using Equation (2-6).

### 2.2.3 Determine the cell shape factor

After the linear electrokinetic mobility measurement, 10 \( \mu \)L of cell suspension was dropped on a glass slide immediately, air dried and observed under a high magnification optical microscope (Figure 2.2). Cells for each bacterial strain were fitted into ellipsoids, and their major and minor semi-axes (\( a \) and \( b \)) were measured using ImageJ. Substituting the DEP mobility data and cell dimensions into Equation (2-7) gives the Clausius-Mossotti factor, \( \kappa_{\text{CM}} \).
Chapter 3

Microbial Cell Envelope Polarizability Correlates with Electrochemical Activity


3.1 Extracellular electron transfer

Extracellular electron transfer (EET) [28, 75] is the capacity for microbes to transfer electrons between their interior and external electron donors or acceptors during anaerobic respiration. It empowers cell growth and/or maintenance of exoelectrogens and electrotrophs, and makes them versatile for multiple applications including environmental remediation [28], microbial fuel cells (MFC) [76, 77], and microbial electrosynthesis [78, 79]. Microbial EET mechanisms have been explored using a number of dissimilatory metal-reducing bacteria (DMRB), among which *Geobacter* and *Shewanella* are the most studied. For example, *Geobacter sulfurreducens* uses a network of multiheme cytochromes [76, 80, 81] to transfer electrons, while *Shewanella oneidensis* uses different sets of proteins, forming a metal-reducing (Mtr) pathway [82], to route electrons across the cell envelope. Moreover, *G. sulfurreducens* can form extracellular conductive pili [83], and *S. oneidensis* can produce outer-membrane and periplasmic extensions [84, 85] that may enable long-distance electron transport. Extensive genetic and biochemical analysis has substantially enhanced our understanding of the EET pathway in a few well-established model microorganisms, and hastened the improvement of their related biotechnological applications. However, key knowledge gaps still remain, partially due to the fact that phenotyping techniques for EET investigations lag behind the development of genotyping methods. Although at least 111
putative \(c\)-type cytochromes have been reported for \(G. \text{sulfurreducens}\) by complete genome sequencing [86], only a few have been fully understood in their phenotype-genotype relationships and physiological functions [80, 81, 87]. Conventional phenotyping techniques to evaluate microbial EET includes cell growth in various conditions [87], measurement of redox products (e.g. \(\text{Fe(II)}\) and \(\text{Mn(III)}\) concentrations) [87-90], and power output in MFCs [76, 80, 91-93]. These phenotyping techniques are time consuming and require large sample volumes, impeding the investigation of difficult-to-culture or slow-growing microorganisms. Rapid and precise phenotyping strategies for microbial EET are imperative to uncover the phenotype-genotype relationship and to select superior candidates for optimized production in microbial electrochemical systems. Recently the electrical conductivity of individual \(G. \text{sulfurreducens}\) pili [94] and electrode-grown biofilms [95, 96] have been measured, where \(G. \text{sulfurreducens}\) components/networks were treated as electronic materials. Compared to the traditional biochemical analysis, these electrical phenotyping methods provide important parameters for \(G. \text{sulfurreducens}\) EET modeling, and suggest the possibility to quantify EET using intrinsic physical properties of microbes.

In this Chapter, the 3DiDEP approach was applied towards electrical phenotyping for several species of DMRB. We demonstrate that microbial EET (a cellular physiological property) is correlated with cell surface polarizability (an electrical property) that can be easily measured by microfluidic systems utilizing 3DiDEP. This work is the first to show the strong correlation between bacterial EET and cell surface polarizability. Our analysis of wild type (WT) \(G. \text{sulfurreducens}\) DL-1 and various cytochrome-deletion mutants shows for the first time that deficiency in expressing \(c\)-type outer-membrane cytochromes known to be responsible for EET measurably reduces cell envelope polarizability. Similar correlations were found with \(S. \text{oneidensis}\) and \(E. \text{coli}\) heterologously expressing \(S. \text{oneidensis}\) EET pathways. Moreover, we show that the decrease of \(S. \text{oneidensis}\) polarizability due to loss of EET pathways can be recovered by reintroducing the EET pathway. Additionally, activation of the microbial EET pathway by switching electron acceptors from pure fumarate to an MFC anode (for \(G. \text{sulfurreducens}\) DL-1) or \(\text{Fe(III)}\) citrate (for \(S. \text{oneidensis}\) strains) enhances cell surface polarizability.
3.2 Correlating *G. sulfurreducens* polarizability with electrochemical activity

A set of proteins, particularly *c*-type outer-membrane cytochromes localized on the cell surface (Figure 3.1a), are known to regulate electron flow across the cell envelope of *G. sulfurreducens*. [81, 87, 89, 92] To quantify a possible correlation between *G. sulfurreducens* electrochemical activity and cell surface polarizability, DL-1 and various cytochrome-deletion mutants (kindly provided by Prof. Derek Lovley at UMass Amherst) were grown with the soluble electron acceptor fumarate, and then were evaluated using the 3DiDEP device. Fumarate was selected as the electron acceptor because the OmcB deficient strains grow as well as the wildtype strain when reducing fumarate, but their growth is diminished with Fe(III) citrate, and even not sustainable when reducing Fe(III) oxide. [87] Fumarate-grown *G. sulfurreducens* DL-1 has been confirmed to express outer-membrane cytochromes OmcB, OmcE, OmcS, OmcT, and OmcZ by several previous studies. [81, 87, 89-91] Additionally, to examine if 3DiDEP is sensitive to detect cell surface phenotypic changes induced by alteration of growth conditions, we further compared cell polarizability of fumarate-grown DL-1 and DL-1 harvested from an MFC anode with varying incubation times. Two MFC strains were measured, one of which was directly inoculated from the fumarate-grown DL-1 cell cultures, and thus required long-term MFC incubation to adapt to the transition to reducing soluble/insoluble electron acceptors. As a further proof of the possible correlation between cell polarizability and MFC incubation, we analyzed another better adapted MFC strain, which was inoculated from DL-1 kept with the insoluble electron acceptor, Fe(III) oxide. The *Geobacter* strains studied are summarized in Table 3.1.

3.2.1 Growth conditions of *G. sulfurreducens*

*G. sulfurreducens* strain DL-1 and cytochrome-deletion mutants were cultured from frozen stocks, inoculated into and propagated once into liquid growth medium following Coppi, et al. [97] The growth medium was supplemented with 10 mM acetate and 40 mM fumarate as the electron donor
and acceptor, respectively, as well as Wolf’s vitamin and mineral supplement (ATCC). The medium (final pH = 6.8) was degassed for 30 min/L at 80 °C in the anaerobic chamber and transferred to glass pressure tubes with butyl stoppers unless otherwise noted. Cells were cultured at 30 °C until reaching early stationary phase (c.a. 3 days). Anaerobic culturing, growth media, and transfers were conducted in an anaerobic chamber (Coy Lab Products) under 5:20:75 H₂ : CO₂ : N₂.

Table 3.1 *G. sulfurreducens* strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relative genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT DL-1</td>
<td><em>G. sulfurreducens</em> strain DL-1, wild type</td>
<td>Leang et al. (2003) [87]</td>
</tr>
<tr>
<td>ΔomcB</td>
<td>WT DL-1 strain without omcB</td>
<td>Leang et al. (2003) [87]</td>
</tr>
<tr>
<td>ΔomcZ</td>
<td>WT DL-1 strain without omcZ</td>
<td>Nevin et al. (2009) [91]</td>
</tr>
<tr>
<td>ΔomcBS</td>
<td>WT DL-1 strain without omcB/omcS</td>
<td>Voordeckers et al. (2010) [98]</td>
</tr>
<tr>
<td>ΔomcBST</td>
<td>WT DL-1 strain without omcB/omcS/omcT</td>
<td>Voordeckers et al. (2010) [98]</td>
</tr>
<tr>
<td>ΔomcBEST</td>
<td>WT DL-1 strain without omcB/omcE/omcS/omcT</td>
<td>Voordeckers et al. (2010) [98]</td>
</tr>
<tr>
<td>ΔomcBESTZ</td>
<td>WT DL-1 strain without omcB/omcE/omcS/omcT/omcZ</td>
<td>Voordeckers et al. (2010) [98]</td>
</tr>
</tbody>
</table>

For the MFC strain, fumarate-grown DL-1 was cultivated in the anode of a microbial fuel cell (MFC) with graphite block electrodes. An H-cell (Adams & Chittenden Scientific Glass) was used as the reactor. The volume of each chamber of the H-cell is 100 mL. A Nafion® N117 membrane (Chemours) was boiled in DI water and then inserted between the two chambers. A 100 mL culture was grown to mid-log phase with fumarate, centrifuged and used to inoculate the anode chamber. The anode chamber contained 100 mL of the growth medium without fumarate. The cathode contained 100 mL of the growth medium lacking fumarate or acetate but included 50 mM potassium ferricyanide as the electron acceptor. The graphite block electrodes were connected by a titanium wire through a 1 kΩ resistor. Current and voltage of the MFC were monitored periodically using an EX430 MultiMeter (Extech Instruments). The bacterial cells were harvested from the anode (with a 16.6 cm² surface area) inside the anaerobic chamber when the MFC approached a current density higher than 45 mA/m². The cells were scrapped off the anode surface using a cell scraper (FisherScientific) and suspended in 1.5 mL of their native growth medium. The cell suspension was then prepared following the method described in Chapter 2.2.1 Section II.
3.2.2 Assessment of *G. sulfurreducens* polarizability

The trapping voltages for the onset of 3DiDEP immobilization (the x-axis data in Figure 3.1b) were measured for the strains and utilized to determine the local critical electric field ($\bar{E}$) by numerical simulation. The numerically estimated electric field leads to the ratio between the magnitudes of DEP mobility versus linear electrokinetic mobility (the y-axis data in Figure 3.1b), $|\mu_{\text{DEP}} / \mu_{\text{EK}}|$, which is inversely proportional to the trapping voltage. The trapping voltage measured for the fumarate-grown DL-1 is significantly distinguished ($p < 0.05$) from that of the fumarate-grown mutants deficient in expressing the outer-membrane cytochrome OmcB and the DL-1 strain grown in an MFC for 31 days. According to Equation (2-6) and (2-7), the trapping voltage is a function of three parameters, including cell surface polarizability ($\kappa_{CM}$), linear

![Figure 3.1 Dielectrophoretic phenotyping of *Geobacter sulfurreducens*. a) A schematic showing that *G. sulfurreducens* c-type outer-membrane cytochromes mediate extracellular electron transfer (EET). b) Measured trapping voltage was plotted against the ratio of DEP mobility ($\mu_{\text{DEP}}$) to the magnitude of linear electrokinetic mobility ($\mu_{\text{EK}}$) of wild type (WT) *G. sulfurreducens* DL-1, DL-1 inoculated in an MFC anode for 24 and 31 days, and various indicated cytochrome-deletion mutants. Significant difference ($p < 0.05$) was found between groups isolated by dashed circles using a Kruskal-Wallis test. The black line indicates the inverse relationship between the ratio $|\mu_{\text{DEP}} / \mu_{\text{EK}}|$ and the applied voltage.](image-url)
electrokinetic mobility \( (\mu_{EK}) \), and cell morphology \((b\text{ and }\xi)\). We measured linear electrokinetic mobility and cell dimensions separately to decouple their effects.

Linear electrokinetic mobilities (Figure 3.2a), \( \mu_{EK} \), were obtained by tracking cell trajectories in straight microfluidic channels under DC electric fields with Particle Image Velocimetry. [74] Removing outer-membrane cytochromes and MFC inoculation did not induce significant difference in measured linear electrokinetic mobilities (Figure 3.2a), suggesting small variations of zeta potentials (or surface charge conditions) at the cell/media interface, given that the electrophoretic mobility scales with zeta potential \( (\zeta) \) as \( \mu_{EK} \sim \frac{\varepsilon_m \zeta}{\eta} \). One hypothesis is that surface charges can be conferred by other cell components including the lipopolysaccharide, which is more abundant on the cell surface compared to outer-membrane cytochromes. Another explanation posits that \textit{G. sulfurreducens} is able to express and use alternative cytochromes when some are unavailable, which may compensate for the variations in cell surface charges.

DEP mobilities of these strains (Figure 3.2b), \( \mu_{DEP} \), were derived from the ratio \( |\mu_{DEP} / \mu_{EK}| \) and measured linear electrokinetic mobilities according to Equation (2-6). Compared to cell surface polarizability, DEP mobility captures both cellular surface dielectric properties and cell shape information. Although the genetic changes made to the cell envelope and the change in growth conditions can lead to some discrepancies in cell major and minor semi-axis (Figure 3.2c and d), these variations have no significant influence on \textit{G. sulfurreducens} polarizability. Cell morphology can affect the cell motion by 1) altering the drag force via the Perrin friction factor, \( \xi \) (Equation 2-3); and 2) changing the DEP force, which depends on the short semi-axis, \( b \) (Equation 2-4). The ratio \( \frac{\xi}{b^2} \) (Figure 3.2e) indicates how much the ellipsoidal cell shape influences the drag force versus the DEP force. Since the DEP mobility for a spherical particle with a radius \( r \) is given by

\[
\mu_{DEP-sphere} = \frac{r^2 \varepsilon_m \Kappa_{CM}}{3\eta},
\]

(3-1)
Figure 3.2 *G. sulfurreducens* electrokinetics and cell morphology. a) Linear electrokinetic mobility (mean +/- std.), $\mu_{EK}$, of the studied nine strains of *G. sulfurreducens*. b) DEP mobility, $\mu_{DEP}$, of the studied nine strains of *G. sulfurreducens*. Pairwise comparison using two-sample $t$-test (two tailed) shows significant difference ($p < 0.03$) between groups not sharing letters (italic bold). c-e) Box-whisker plots of bacterial major semi-axis (c), minor semi-axis (d) and the ratio of Perrin friction factor to the square of cell short semi-axis $\xi/b^2$ (e) by ellipsoidal fit for the nine investigated *G. sulfurreducens* strains indicate median and interquartile (IQR) ranges. The whiskers extend to 1.5IQR below the 25th percentile and above the 75th percentile, respectively. Blank dots indicate the outliers. Asterisks indicate significant difference ($p < 0.01$) compared to the control (WT DL-1) by a Kruskal-Wallis test. The number of measured cells ($n$) are 150, 100, 100, 244, 100, 445, 238, 50, and 100, respectively, following the order in panel (a). Colors in all panels correspond to the legend in Figure 3.1b.
physically (with Equation 2-7) $b / \sqrt[3]{\xi}$ can be considered as the equivalent DEP radius for an ellipsoidal particle. No significant difference was found for the ratio $\xi / b^2$ among the investigated *G. sulfurreducens* strains (Figure 3.2e), and thus their DEP mobility (Figure 3.2b) and the Clausius-Mossotti factor (Figure 3.3) follow a similar trend.

The Clausius-Mossotti factors represent the surface polarizabilities of the *G. sulfurreducens* strains, and were estimated according to Equation (2-7). Many authors report Clausius-Mossotti factor being restricted from negative 0.5 to 1, but this is done assuming homogeneous spherical particles. However, the Clausius-Mossotti factor of bacteria can be higher than 1 (see Figure 3.11) due to multiple physiological features such as their non-spherical shapes [67] and/or charged soft extracellular layers [99], e.g. LPS and pili produced by *G. sulfurreducens* (see Chapter 3.5).

### 3.2.3 Effects of c-type outer-membrane cytochromes on *G. sulfurreducens* polarizability

Our results indicate that removing c-type outer-membrane cytochromes can lead to decreased cell surface polarizability in *G. sulfurreducens* (Figure 3.3). Comparing the seven *G. sulfurreducens* strains grown with fumarate suggests a ranking of cell surface polarizability ($p < 0.02$) as WT $\approx$ $\Delta$omcZ $> \Delta$omcB $\approx \Delta$omcBS $\approx \Delta$omcBST $\approx \Delta$omcBEST $> \Delta$omcBESTZ (Figure 3.3). In particular, the $\Delta$omcBESTZ quintuple mutant displayed a 70% decrease in cell surface polarizability compared to strain DL-1. Moreover, removing different genes encoding outer-membrane cytochromes results in distinct effects on *G. sulfurreducens* surface polarizability. As shown in Figure 3.3, all the mutants missing gene omcB ($\Delta$omc, $\Delta$omcBS, $\Delta$omcBST, $\Delta$omcBEST, and $\Delta$omcBESTZ) show significantly suppressed polarizability ($p < 0.02$) compared to strain DL-1. Removing OmcB alone induces a decrease in cell surface polarizability by a factor of two ($\Delta$omcB vs. DL-1 in Figure 3.3), suggesting a substantial impact of OmcB on *G. sulfurreducens* surface polarizability. However, removing OmcZ alone did not change cell polarizability significantly ($\Delta$omcZ vs. DL-1 in Figure 3.3), while simultaneous deletion of OmcBESTZ induces a further decrease in cell surface polarizability compared to the quadruple mutant $\Delta$omcBEST ($\Delta$omcBEST
Figure 3.3 *G. sulfurreducens* cell polarizability is positively correlated with EET capacity. *G. sulfurreducens* polarizability, represented by the Clausius-Mossotti factor ($\kappa_{CM}$), of the nine investigated strains (left y-axis), as well as the current density (blue circles, right y-axis) generated by *G. sulfurreducens* grown in an MFC. Italic bold letters above the bars show the result of pairwise comparison using two-sample $t$-test (two tailed) with the following number of repeats: $n = 3$ (WT DL-1, $\Delta$omcBST), $n = 4$ ($\Delta$omcB, $\Delta$omcZ, $\Delta$omcBS, $\Delta$omcBEST, and $\Delta$omcBESTZ), $n = 5$ (MFC 24 days) and $n = 7$ (MFC 31 days). Significant difference ($p < 0.02$) was found between groups not sharing letters.
vs. ΔomcBESTZ in Figure 3.3). One hypothesized reason for this discrepancy is that OmcZ has a smaller impact on surface polarizability than OmcB, which could be due to their distinct locations within the cell envelope. OmcB is embedded in the outer membrane of *G. sulfurreducens* and partially exposed to the outer surface, [90] while OmcZ is only loosely bound to the outer membrane [81, 91, 92] (Figure 3.1a). Another possible reason is that the single-deletion mutant ΔomcZ is adapted by up-regulating other outer-membrane cytochromes [92] (Table 3.2), which may mask any potential drop in cell surface polarizability. This adaptation capability of *G. sulfurreducens* may also explain the fact that there is no significant difference in cell surface polarizability among the mutants ΔomcB, ΔomcBS, ΔomcBST, and ΔomcBEST. Otherwise, it is possible that the presence of OmcE, OmcS, and OmcT does not have a large effect on *G. sulfurreducens* surface polarizability. *G. sulfurreducens* utilizes different outer-membrane cytochromes for EET when reducing different kinds of electron acceptors. [81, 87-92] For instance, removing OmcB results in significant deficiency for *G. sulfurreducens* to reduce both soluble and insoluble Fe(III), [87, 88, 90] while OmcZ is only essential when an MFC anode serves as the electron acceptor. [81, 91, 92] Despite the adaptation capacity of *G. sulfurreducens*, a number of outer-membrane cytochromes (including OmcB and OmcZ) are not interchangeable in their physiological functions for EET. [88] For example, OmcB-deficient mutants never adapt to grow with Fe(III) oxide, [88] while OmcZ-deficient mutants show severe inhibition in MFC current production with no long-term adaptation.[92] Our observation of distinct cell polarizability between OmcB- and OmcZ- deficient mutants indicates the potential of using microfluidic dielectrophoresis to separate *G. sulfurreducens* based on its activity for reducing different electron acceptors. The physiological roles of the c-type outer-membrane cytochromes investigated in this work have been well studied for various growth conditions, and are summarized in Table 3.2.

### 3.2.4 Effects of MFC incubation on *G. sulfurreducens* polarizability

The MFC strains (in Figure 3.3) were directly inoculated from the fumarate-grown DL-1 cultures. It has been reported that *G. sulfurreducens* adapted to anode-respiring conditions has substantially enhanced EET compared to the inoculum due to up-regulation of a number of c-type outer-membrane cytochromes [91, 92] and pili [91] (Table 3.2). Correspondingly, we observed that the
cell surface polarizability for strain DL-1 is doubled comparing to that of its inoculum after growing in an MFC for 31 days. Moreover, comparing strains DL-1, MFC (24 days), and MFC (31 days) suggests an increasing trend in cell surface polarizability as MFC incubation time increases (Figure 3.3). Our 24-day and 31-day MFC strains achieved a current density of 0.046 A/m² and 0.404 A/m², and a power density of 0.016 W/m² and 0.246 W/m², respectively, suggesting different production in related outer-membrane cytochromes and pili. [91] This indicates that cell surface polarizability is sensitive enough to detect the phenotypic change of *G. sulfurreducens* during growth in an MFC, and further proves that *G. sulfurreducens* EET correlates with cell surface polarizability. Data for strains with an MFC incubation time shorter than 24 days are not provided because the cell concentration was too low (OD600 < 0.05) for DEP-based screening. The MFC strains (in Figure 3.3) show a growth rate slower than some of the reported values, given that their inoculum—the fumarate-grown DL-1—is not immediately adapted to reduce the MFC anode. Additionally, we used an anode area and MFC configuration different from the conditions in the previous studies, which may also explain the difference in growth rate. We also analyzed another anode-respiring DL-1 strain, which was grown with an additional step following the protocol by Bond and Lovley [76]. The cells used were maintained in a medium with Fe(III) oxide to be better adapted to reduce insoluble electron acceptors. Fe(III) oxide particles in the cell cultures were then eliminated by transferring the cells three times in a medium containing 40 mM fumarate prior to inoculation into the MFC. This new MFC strain showed an earlier biofilm formation (~ 1 week) on the anode surface compared to the MFC strain directly inoculated from fumarate-grown cultures (~ 3 weeks), confirming the impact of original acclimation of the bacteria on their growth rate in the MFC. The result for the new MFC strain demonstrates once again that *G. sulfurreducens* surface polarizability increases with the MFC incubation time (Figure 3.4).
Figure 3.4 **MFC incubation time affects *G. sulfurreducens* polarizability.** Trapping voltage (a), Linear electrokinetic mobility (b), DEP mobility (c), and cell polarizability (d) of *G. sulfurreducens* strain DL-1 harvested from an MFC anode with varying incubation time. Different from the growth condition corresponding to Figure 3.3, here cells were inoculated with Fe(III) oxide to be better adapted to reduce insoluble electron acceptors prior to the MFC incubation. Error bars indicate the standard deviation. Two-tailed *t*-test was performed with a sample size of *n* = 4.
Table 3.2 Summary of *G. sulfurreducens* c-type outer-membrane cytochromes in this study and their roles in EET.

<table>
<thead>
<tr>
<th>C-type outer-membrane cytochromes</th>
<th>Location</th>
<th>Electron acceptor</th>
<th>MFC anode</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>OmcB</strong></td>
<td>Tightly associated with the outer membrane, and partially exposed to the extracellular environment.</td>
<td>Crucial for optimal reduction of Fe(III) citrate and Fe(III) oxide. Facilitates electron transfer from the periplasm to the outer surface. Not required for fumarate-respiration (Fumarate reductase is either cytoplasmic or periplasmic, and so outer-membrane cytochromes are not involved). OmcB-deficient mutant never adapts to grow with Fe(III) oxide. Although it can gradually adapt to reduce soluble Fe(III) at a much lower growth rate, the loss of OmcB cannot be fully adapted.</td>
<td>Mediates heterogeneous EET across the biofilm/electrode interface. Expression level of omcB gene is significantly increased. Deletion of omcB has no impact on maximum current production.</td>
<td>[87, 88, 90-92]</td>
</tr>
<tr>
<td><strong>OmcE</strong></td>
<td>Exposed on the outside of the cell.</td>
<td>Exclusively required for Fe(III) oxide reduction, but not for Fe(III) citrate. OmcE-deficient mutant gradually adapts to reduce Fe(III) oxide after 30 days of inoculation.</td>
<td>Not directly involved. The expression level of omcE is increased. Deletion of omcE has no impact on maximum current production.</td>
<td>[89, 92]</td>
</tr>
<tr>
<td><strong>OmcS</strong></td>
<td>Exposed on the outside of the cell, along the conductive pili.</td>
<td>Facilitates electron transfer from the pili to Fe(III) oxide. Not required for Fe(III) citrate reduction (the omcS gene is not expressed).</td>
<td>Plays a secondary role in homogeneous EET. The expression level is down-regulated. Deletion of omcS has no impact on maximum current production.</td>
<td>[89, 91, 92, 100]</td>
</tr>
<tr>
<td><strong>OmcT</strong></td>
<td>Loosely bound to cell outer surface, with a negligible abundance compared to OmcS.</td>
<td><em>OmcT</em> is immediately downstream of the <em>omcS</em> gene. Deleting either <em>omcS</em> or <em>omcT</em> negatively impacts expression of the other cytochrome gene. The presence of OmcT is not sufficient for Fe(III) oxide reduction.</td>
<td>Plays a secondary role in homogeneous EET. Expression of <em>omcT</em> is down-regulated.</td>
<td>[89, 91, 101]</td>
</tr>
</tbody>
</table>

---

a) Here describes *G. sulfurreducens* EET through relatively thick (ca. 50 µm) biofilms to MFC anodes, with high-density current production. Mechanisms for this long-range EET differ from that in the case where most of the cells are in direct contact with the electron acceptors.
A number of studies show that *G. sulfurreducens* can adapt to electrode respiration over time for enhanced efficiency in current production on a per cell basis. [81, 91-93, 102] An isolate, designated strain KN400 by Yi et al. [93], recovered from *G. sulfurreducens* biofilms inoculated 5 months in an MFC has a superior capacity for current production compared to its original inoculum [103]; it grows more rapidly, generates higher maximum current, and requires fewer cells. Compared to DL-1, strain KN400 has been shown to utilize more of the electron flow for current production rather than cell synthesis, and the enhanced current production has been associated with a number of phenotypic changes in cell outer surface. [93] Additionally, cyclic voltammetry of anode-respiring *G. sulfurreducens* has shown that the maximum biofilm thickness and current production are predominantly limited by cell EET rather than the mass transport of reactants. [103] This suggests opportunities for evolutionary selection of *G. sulfurreducens* (and other electrochemically active microorganisms) for optimal current production using cell surface polarizability as a proxy.
3.3 *S. oneidensis* polarizability is positively correlated with EET

To explore if the correlation between cell surface polarizability and microbial EET is generalizable to other electrochemically active microorganisms and other growth conditions, we investigated *S. oneidensis*, a facultative anaerobe that uses a different EET pathway than *G. sulfurreducens*. EET in *S. oneidensis* requires the Mtr respiratory pathway, which consists of a periplasmic c-type cytochrome (e.g. MtrA or MtrD), an integral β-barrel protein located in the outer-membrane (e.g. MtrB or MtrE), and an outer-membrane decaheme c-type cytochrome (e.g. MtrC, MtrF or OmcA). [82, 104] These components cooperatively facilitate electron transfer from the periplasm of the cell to the extracellular electron acceptors (Figure 3.5a). Since these Mtr components show various activities in iron reduction, we analyzed five *S. oneidensis* strains (Table 3.3) to address the following three questions: (i) is the correlation between cell polarizability and EET generalizable to *Shewanella* strains; (ii) is dielectrophoretic screening sufficiently sensitive to distinguish cell envelope phenotypic variations induced by altering different Mtr pathways; and (iii) is this correlation affected by change of growth conditions? To address these three questions, we obtained five *S. oneidensis* strains from Prof. Jeffrey Gralnick at University of Minnesota. The five strains investigated are the wild type strain MR-1, a *S. oneidensis* strain deficient in iron reduction by knocking out all genes identified in the Mtr pathway (ΔMtr), and three complemented strains including various combinations of Mtr components, namely, the ΔMtr strain complemented with *mtrABC, mtrDEF*, and the empty vector pBBR-BB [82].

Table 3.3 *S. oneidensis* strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relative genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT MR-1</td>
<td><em>S. oneidensis</em> strain MR-1, wild type</td>
<td>Coursolle and Gralnick (2012) [82]</td>
</tr>
<tr>
<td>ΔMtr</td>
<td>ΔmtrB/ΔmtrE/ΔmtrC/ΔomcA/ΔmtrF/ΔmtrA/ΔmtrD/ΔdmsE/ΔSO4360/ΔcctA/ΔrecA</td>
<td></td>
</tr>
<tr>
<td>ΔMtr + MtrABC</td>
<td>ΔMtr strain with plasmid pmtrB/mtrC/mtrA</td>
<td></td>
</tr>
<tr>
<td>ΔMtr + MtrDEF</td>
<td>ΔMtr strain with plasmid pmtrE/mtrF/mtrD</td>
<td></td>
</tr>
<tr>
<td>ΔMtr + vector</td>
<td>ΔMtr strain with pBBR-BB</td>
<td></td>
</tr>
</tbody>
</table>
3.3.1 Growth conditions of *S. oneidensis*

*S. oneidensis* strain MR-1 and mutants were inoculated from frozen stocks and grown in LB broth aerobically at 30 °C, 200 rpm shaking for 16 hours, and then transferred 1:100 and grown anaerobically at 30 °C in 20 mL *Shewanella* basal media (SBM) [105] supplemented with 100 mM HEPES, 0.2% casamino acids, Wolf’s vitamin and mineral supplement (ATCC) for ca. 10 hours. The anaerobic growth media (final pH = 7.0) also contained either 10 mM lactate and 60 mM fumarate (growth condition i) or 20 mM lactate and 15 mM Fe(III) citrate supplemented with 10 mM fumarate (growth condition ii). Kanamycin was also provided at a concentration of 50 µM/mL for the growth of ΔMtr complementary mutants (ΔMtr + MtrABC, ΔMtr + MtrDEF, and ΔMtr + vector). The cell suspension was then prepared following the method described in Chapter 2.2.1 for 3DiDEP measurement.

3.3.2 Iron reduction of *S. oneidensis*

The Fe(II) concentration was determined with ferrozine assay adapted from Stookey [106]. Anaerobic cultures of *S. oneidensis* strains were grown with Fe(III) citrate and fumarate, under growth condition ii described above. The purpose of supplementing a small amount of fumarate is to ensure the growth of all strains. At each time point, one aliquot of each culture was centrifuged at 10,000 rpm for 5 min in the anaerobic chamber to pellet the cells, and 100 µL the supernatant was acid extracted in 900 µL 0.5 M hydrochloric acid (HCl) to yield concentrations within the range of standard curves. The total iron concentration was determined by a separate acid extraction with 10% hydroxylamine hydrochloride (HAHC) in 0.5 M HCl for 24 hours. A hundred microliter of each acid extracted sample was mixed with 900 µL ferrozine reagent, which absorbs at 562 nm when chelating Fe(II). The ferrozine reagent contains 10 mM ferrozine (Sigma-Aldrich) in 500 mM HEPES (final pH = 7.0 adjusted by 2N NaOH). The absorbance of all samples was recorded at 562 nm with a UV Vis spectrophotometer (SHIMADZU, Japan) and was used to determine the formation of Fe(II) over time. The Fe(II) concentration in each culture was subtracted by abiotic iron reduction observed in medium-only controls at each time point. Standard curves were made from ferrous sulfate dissolved in 0.5 N HCl.
We measured iron reduction rate of the five strains. As expected, the ΔMtr strain and the strain with the empty vector are defective in Fe(III) citrate reduction (Figure 3.5b). MR-1 has the highest iron reduction rate, followed by the complemented strain expressing mtrABC, which shows roughly twice the reduction rate compared to that of the complemented strain expressing mtrDEF (Figure 3.5b). Our results suggest that the mtrABC paralog is superior to mtrDEF in terms of Fe(III) citrate reduction, agreeing with previous observations [82].

![Diagram of Shewanella oneidensis cell envelope and iron reduction](image)

Figure 3.5 *Shewanella oneidensis* cell envelope and iron reduction. (a) A schematic of the Mtr EET pathway in *S. oneidensis* cell envelope. (b) Fe(III) citrate reduction over time measured for *S. oneidensis* wild type strain MR-1, strain deficient in expressing both MtrABC and MtrDEF EET conduits (ΔMtr), and ΔMtr complemented with indicated proteins. Error bars indicate the standard deviation.

### 3.3.3 Effects of different EET pathways and growth conditions on *S. oneidensis* polarizability

We then performed dielectrophoretic screening with these five *S. oneidensis* strains grown in both condition (i) and (ii). In growth condition (i), fumarate is the only electron acceptor, whereas in growth condition (ii), Fe(III) citrate is the major electron acceptor Figure 3.5b. Figure 3.6 shows
the measured trapping voltage and linear electrokinetic mobilities for the five strains in both growth conditions. No significant statistical difference ($p > 0.05$) was observed in linear electrokinetic mobilities, suggesting that alternating EET pathways or electron acceptors do not result in important effects on cell zeta potential/surface charge conditions. As shown in Figure 3.6c, removing the Mtr pathways leads to a significant drop in $S. oneidensis$ DEP mobility ($\Delta$Mtr vs. MR-1). In growth condition (i), this drop in DEP mobility is reversible by adding the MtrABC EET conduit, but not the MtrDEF EET conduit ($\Delta$Mtr+MtrABC vs. $\Delta$Mtr+MtrDEF). However, in growth condition (ii), adding MtrABC and MtrDEF EET conduits (Figure 3.6cii) both increase the DEP mobility to a level comparable to the case of adding MtrABC EET conduit in growth condition i (Figure 3.6ci). Additionally, adding iron to the growth medium increases the DEP mobility of WT MR-1 but not the $\Delta$Mtr strain.

Figure 3.7 shows the effects of removing EET pathways and changing growth conditions on cell morphology. Although the mutant strains show lower values of $\xi/b^2$ compared to WT MR-1 ($p < 0.01$), and the presence of iron in the growth medium leads to lower values of $\xi/b^2$ ($p < 0.001$) in WT-MR-1, $\Delta$Mtr and $\Delta$Mtr+vector strains (Figure 3.7), these changes in cell shape is not sufficient to alter the trend in cell surface polarizability (Figure 3.8).

In both growth condition (i) and (ii), deletion of Mtr pathways lowered $S. oneidensis$ polarizability ($\Delta$Mtr vs. MR-1 in Figure 3.8), and this change is reversible by adding EET conduits, demonstrating a strong correlation between cell polarizability and EET in $S. oneidensis$. When reducing fumarate, the drop of $S. oneidensis$ polarizability is recovered by adding the MtrABC EET conduit as opposed to the MtrDEF EET conduit (Figure 3.8i), suggesting that the level of $S. oneidensis$ polarizability can (at least partially) be attributed to the presence of MtrABC. Fumarate respiration in $S. oneidensis$ occurs only in the periplasm of the cell, i.e. the outer-membrane components in the Mtr pathway are not involved in condition (i). However, all metal reduction occurs extracellularly [82]. Thus, in the second condition, all the EET components in the Mtr pathway are involved to reduce Fe(III) citrate.
Figure 3.6 *S. oneidensis* electrokinetics. Trapping voltage (a), Linear electrokinetic mobility (b), and DEP mobility (c) of *S. oneidensis* wild type strain MR-1, strain deficient in expressing both MtrABC and MtrDEF EET conduits (∆Mtr), and ∆Mtr complemented with indicated proteins (Table 3.3) grown with different electron accepters, namely, (i) 60 mM fumarate and (ii) 15 mM Fe(III) citrate supplemented with a small amount (10 mM) of fumarate. Bold letters above the box plots indicate results of a multiple comparison test of group means using one-way ANOVA with a significance level of 0.05. Groups sharing a letter suggest no significant difference. Asterisk indicates significant difference (*p* < 0.03, two-tailed *t*-test) between the data of iron-reducing *S. oneidensis* (ii) and that of its fumarate-reducing counterpart (i).
Cell surface polarizabilities measured for the MR-1 and complemented strain expressing mtrDEF (Figure 3.8ii) are both significantly greater than cell surface polarizabilities measured for their fumarate-reducing counterparts (Figure 3.8i), while no statistical difference was observed between the strains complemented with mtrABC grown in the two conditions. Additionally, the ΔMtr strain complemented with mtrDEF shows a significantly stronger polarizability than the mutants ΔMtr and ΔMtr + vector. This comparison suggests that the MtrDEF pathway starts to be involved in the EET process under conditions of excess iron, and the activation of the MtrDEF pathway can also enhance S. oneidensis cell surface polarizability. Comparing the five S. oneidensis strains grown with Fe(III) citrate suggests a ranking of cell polarizability \( p < 0.05 \), WT > ΔMtr + MtrABC ≈ ΔMtr + MtrDEF > ΔMtr + vector > ΔMtr (Figure 3.8ii), which approximately agrees with the ranking of their iron reduction rate (Figure 3.5b).
Figure 3.8 **DEP screening indicates positive correlation between *S. oneidensis* polarizability and EET activity.** Polarizability of the five *S. oneidensis* strains grown with different electron accepters, namely, (i) pure fumarate (60 mM) and (ii) 15 mM Fe(III) citrate supplemented with a small amount (10 mM) of fumarate. The box-whisker plots indicate median and interquartile (IQR) ranges, with whiskers extend to 1.5IQR below the 25\textsuperscript{th} percentile and above the 75\textsuperscript{th} percentile, respectively. Black crosses indicate outliers. Multiple comparison test of group means using one-way ANOVA suggests significant difference (*p* < 0.05) between groups labeled with different letters. Asterisk indicates significant difference (*p* < 0.03, two-tailed *t*-test) between the polarizability of iron-reducing *S. oneidensis* (ii) and that of its fumarate-reducing counterpart (i).
We further investigated the cell polarizability of *E. coli* heterologously expressing an Mtr respiratory pathway from *S. oneidensis*. Heterologous expression of CymA and MtrABC (localization as depicted in Figure 3.5a) has been achieved by co-transforming the plasmid *cymAmtrCAB* with the cytochrome *c* maturation (*ccm*) plasmid into *E. coli* strain C43, enabling extracellular electron transfer in *E. coli*. [107] CymA is the *c*-type cytochrome anchored in the cytoplasmic membrane that donates electrons to a variety of respiratory pathways spanning the periplasm and outer membrane of *S. oneidensis*. [82, 107] Two *E. coli* strains, the control strain (*ccm*) and the electrogenic *E. coli* strain expressing the MtrABC pathway (*ccm+CymA/MtrABC*), are kindly provided by Dr. Caroline Ajo-Franklin at Lawrence Berkeley National Laboratory. Previous studies have confirmed the expression and redox activity of CymA, MtrA, and MtrC in the electrogenic *E. coli* strain. [107]

*E. coli* strains *ccm* and *ccm+CymA/MtrABC* were cultured from frozen stocks and grown aerobically overnight in 2xYT medium at 37 °C, 250 rpm shaking, and then transferred 1:100 into 25 mL 2xYT medium and grown with 250 rpm shaking for 16 hours at 30 °C. Then, each strain was centrifuged at 6,000 rpm for 4 min and resuspended (with an OD600 ~ 0.7) in 20 mL of the anaerobic M1 medium [107] supplemented with 0.2% casamino acids, 40 mM lactate, 15 mM Fe(III) citrate, and 10 mM fumarate, and grown in the anaerobic chamber for five days at 30 °C. The growth medium also contains 50 µM/mL Kanamycin and 30 µM/mL chloramphenicol. Iron reduction measurement was performed following the method in Section 3.3.2.

As shown in Figure 3.9, no significant difference was observed between linear electrokinetic mobilities of the electrogenic *E. coli* strain and the control strain. The electrogenic *E. coli* strain shows a stronger DEP mobility compared to the control. Although the introduction of the *Shewanella* EET pathway decreases the cell equivalent DEP radius (Figure 3.9f), $b/\sqrt{\xi}$, this change in cell shape is not sufficient to induce a change in the trend of cell surface polarizability.
Figure 3.9 **Electrogenic *E. coli* electrokinetics and cell morphology.** a)-c) Trapping voltage (a), Linear electrokinetic mobility (b), and DEP mobility (c) of the *E. coli* strain transformed with an empty cytochrome *c* maturation (*ccm*) plasmid (control) and the strain co-transformed with *S. oneidensis* MtrABC EET conduit grown with 15 mM Fe(III) citrate and 10 mM fumarate (two-tailed *t*-test). d)-f) Box-whisker plots of bacterial major semi-axis (d), minor semi-axis (e) and the ratio of Perrin friction factor to the square of cell short semi-axis $\xi/b^2$ (f) by ellipsoidal fit for the investigated *E. coli* strains (two-tailed *t*-test). Numbers in pane (d) indicate the number of measured cells ($n$).
Figure 3.10 *Escherichia coli* introduced with EET pathways from *Shewanella oneidensis* gains strong polarizability. (a) Polarizability of the *E. coli* strain transformed with an empty cytochrome *c* maturation (*ccm*) plasmid (control) and the strain co-transformed with *S. oneidensis* MtrABC EET conduit grown with 15 mM Fe(III) citrate and 10 mM fumarate. The electrogenic *E. coli* strain obtains significantly enhanced polarizability (*p < 0.0001*, two tailed *t*-test, *n* = 8) compared to the control. b) Fe(III) citrate reduction over time measured for the control and electrogenic *E. coli* strain. c) Positive relationship between bacterial polarizability and iron reduction rate of the studied five *S. oneidensis* strains and two *E. coli* strains is indicated by a log fitting (dashed line) with a fitting goodness of $R^2 = 0.91$. The iron reduction rate was derived by taking the slope of the linear portion of the Fe(III) citrate reduction curves.
Our dielectrophoretic screening shows that the electrogenic *E. coli* strain has a significantly stronger surface polarizability ($p < 0.0001$) compared to the ccm strain (Figure 3.10a). This result provides further evidence that the presence of the MtrABC pathway enhances cell surface polarizability, regardless of the species of the microbe. The electrogenic *E. coli* strain reduces Fe(III) citrate ~ 3.5x faster than the ccm strain (Figure 3.10b), consistent with the results of previous studies [107], suggesting a positive correlation between iron reduction and cell surface polarizability. Figure 3.10c plots the cell surface polarizability of the five *S. oneidensis* strains and two *E. coli* strains grown with Fe(III) citrate against their iron reduction rates. It suggests that *S. oneidensis* has a superior iron reduction activity and cell polarizability compared to the electrogenic *E. coli*. It also suggests that 3DiDEP can be utilized to distinguish microbes from different species based on their iron reduction activity (or other phenotypes related to redox activity), though species may differ in their baseline cell polarizability.

### 3.5 Calculation of the Clausius-Mossotti factor for two-shelled ellipsoidal particles

In this Section, we use a two-shelled prolate ellipsoid modal [67] to verify two hypotheses: 1) the Clausius-Mossotti factor for bacteria can be higher than one; and 2) the Clausius-Mossotti factor measured by 3DiDEP is dominated by cell surface (rather than internal) properties under DC electric fields.

Many authors report Clausius-Mossotti factor being restricted from -0.5 to 1, but this is done assuming homogeneous spherical particles, which obviously disaccord with the facts of bacteria. A more reasonable simplification is to assume the bacterial cell as an ellipsoid of highly conductive cytoplasm enclosed by two concentric less conductive membranes with constant thickness (Figure 3.11a), and estimate the Clausius-Mossotti factor in three steps by implementing the Maxwell and Wagner theories at each interface of the neighboring layers. [67]
Figure 3.11 **The Clausius-Mossotti factor of bacteria can be higher than one.** a) A schematic of the two-shelled ellipsoidal model for bacteria (not drawn to scale). The bacterial cytoplasm is described as an ellipsoid with a major semi-axis $a_0$, minor semi-axis $b_0$, surrounded by an inner membrane layer of thickness $d_{IM}$ and cell wall of thickness $d_w$. $\sigma_{cyto}$, $\sigma_{IM}$, $\sigma_w$, and $\sigma_m$ are conductivities of the cytoplasm, inner membrane, cell wall, and the surrounding media, respectively. b) Clausius-Mossotti factor ($\kappa_{CM}$) versus the ratio between conductivities of cell wall and surrounding media ($\sigma_w / \sigma_m$) estimated by the two-shelled ellipsoidal model using Equation 3-9 (solid line) and the homogeneous spherical model (dashed line).
Starting with the interface of the cytoplasm core and the inner membrane (IM) layer (Figure 3.11a), the effective dipole factor along the major axis of the ellipsoid can be expressed as

\[ \kappa_1 = \frac{1}{3} \frac{e_{cyto}^* - e_{IM}^*}{e_{IM}^* + A_1(e_{cyto}^* - e_{IM}^*)} \]  

(3-2)

where \( A_1 \) is the depolarization factor along the major axis of the ellipsoid given by

\[ A_1 = \frac{1 - e_1^2}{2e_1^3} \left[ \log \left( \frac{1 + e_1}{1 - e_1} \right) - 2e_1 \right] \]  

(3-3)

and the eccentricity is expressed by the major (\( a_0 \)) and minor (\( b_0 \)) semi-axes of the cytoplasm core (Figure 3.11a) as

\[ e_1 = \sqrt{1 - \left( \frac{b_0}{a_0} \right)^2} \]  

(3-4)

Under DC electric fields, the complex permittivity of cytoplasm (\( e_{cyto}^* \)) and the inner membrane (\( e_{IM}^* \)) in Equation 3-2 can be replaced by their corresponding conductivities (\( \sigma_{cyto} \) and \( \sigma_{IM} \)), respectively. The inner membrane is a phospholipid bilayer, and has a conductivity over three orders of magnitudes lower than the conductivity of cytoplasm for Gram-negative bacteria. [51, 67] Thus the inner membrane shell can be considered as electrically insulating, and Equation 3-2 is simplified as

\[ \kappa_1 = \frac{1}{3A_1} \]  

(3-5)

Then the effective dipole factor in response to DC electric fields at the interface between the inner membrane and the cell wall (Figure 3.11a) is expressed as

\[ \kappa_2 = \frac{1}{3} \frac{\sigma_{IM} - \sigma_w}{\sigma_w + A_2(\sigma_{IM} - \sigma_w) + 3\kappa_1 \rho_1 \left[ \sigma_{IM} + A_2(\sigma_w - \sigma_{IM}) \right] + 3\kappa_1 \rho_1 A_2(1 - A_2) (\sigma_{IM} - \sigma_w)} \]  

(3-6)

Again, the conductivity of the inner membrane (\( \sigma_{IM} \)) is much lower than the conductivity of the cell wall (\( \sigma_w \)), [51, 67] and thus the dipole factor is simplified by
\[
\kappa_2 = \frac{1}{3} \frac{3 \kappa_1 \rho_2 A_2 - 1}{1 - A_2 - 3 \kappa_1 \rho_2 (1 - A_2)} = \frac{1}{3 (A_2 - 1)} \tag{3-7}
\]

where the depolarization factor \( A_2 \) is again defined as in Equation 3-3 except that the eccentricity changes to

\[
e_2 = \sqrt{1 - \left( \frac{b_0 + d_{IM}}{a_0 + d_{IM}} \right)^2} \tag{3-8}
\]

with \( d_{IM} \) being the inner membrane thickness. \( \rho_1 \) is the volume ratio between the cytoplasm core and the ellipsoidal region enclosed by the outer surface of the inner membrane. Finally, the Clausius-Mossotti factor (i.e. the effective dipole factor along the ellipsoidal major axis) is expressed by the conductivity of the cell wall (\( \sigma_w \)) and the surrounding media (\( \sigma_m \)) as

\[
\kappa_{CM} = \frac{1}{3} \frac{\sigma_w - \sigma_m}{\sigma_m + A_3 (\sigma_w - \sigma_m)} + 3 \kappa_2 \rho_2 \left[ \sigma_w + A_3 (\sigma_m - \sigma_w) \right] \tag{3-9}
\]

where the depolarization factor \( A_3 \) is defined as in Equation 3-3 as before except that the eccentricity changes to

\[
e_3 = \sqrt{1 - \left( \frac{b_0 + d_{IM} + d_w}{a_0 + d_{IM} + d_w} \right)^2} \tag{3-10}
\]

and the volume ratio is

\[
\rho_2 = \frac{(a_0 + d_{IM})(b_0 + d_{IM})^2}{(a_0 + d_{IM} + d_w)(b_0 + d_{IM} + d_w)^2} \tag{3-11}
\]

where \( d_w \) is the thickness of the cell wall.

Cell wall for Gram-negative bacteria consists of the outer membrane, which is a lipid bilayer principally composed of lipopolysaccharide (LPS) in its outer leaflet and phospholipids in its inner leaflet (Figure 3.1a and Figure 3.5a). [1] The outer and inner membranes delimit the periplasmic space, an aqueous cellular compartment, which includes networks of peptidoglycan. [1] Unlike the inner membrane (a phospholipid bilayer), the abundance of charged groups (e.g. LPS), ion-
exchangers (e.g. the outer-membrane cytochromes in *G. sulfurreducens*) and electrically conductive features (e.g. pilli) impart a much higher conductivity to the outer membrane compared to that of the inner membrane, which ensures that the Clausius-Mossotti factor measured by 3DiDEP using DC electric fields is dominated by cell surface properties. The ratio between conductivities of the cell surface and surrounding media ($\sigma_w / \sigma_m$) for *G. sulfurreducens* is high, which can result in a Clausius-Mossotti factor higher than 1. For instance, the conductivity of individual WT *G. sulfurreducens* pili is $51 \pm 19$ mS/cm, [94] which is more than 500 times higher than that of the surrounding medium (ca. 0.1 mS/cm) used in this study. Using the cell dimensions averaged over all measured *G. sulfurreducens* strains in this study ($a = 1.1 \mu$m and $b = 0.4 \mu$m) and reported thicknesses of inner membrane (ca. 5 nm) [1, 67] and cell wall (ca. 35 nm, including a 25 nm thick periplasm and 10 nm thick outer membrane), [108] Figure 3.11b (solid line) shows the estimated Clausius-Mossotti factor for various ratios between the conductivities of cell wall and the surrounding medium. Compared to the Clausius-Mossotti factor estimated by the spherical model (dashed line in Figure 3.11b) that is always lower than 1, the ellipsoidal model shows that the Clausius-Mossotti factor can be higher than 1. Additionally, the structure of bacterial outer membrane is far more complex than a homogeneous solid body. [1] The existence of soft polyelectrolyte layers can result in an even higher Clausius-Mossotti factor. [99]

### 3.6 Summary

In this chapter, we demonstrate for the first time the correlation between microbial EET and cell surface polarizability. By comparing the Clausius-Mossotti factor ($\kappa_{CM}$) of *G. sulfurreducens*, *S. oneidensis*, and electrogenic *E. coli* strains in different growth conditions, we show that microbial EET can be distinguished by cell surface polarizability using 3DiDEP, and the correlation is generalizable to multiple species. The level of cell surface polarizability is contingent on the amount of crucial outer-membrane cytochromes and the integrity of EET pathways, e.g. high polarizability was found in both wild type *G. sulfurreducens* and *S. oneidensis* (versus their cytochrome-deletion mutants) and *E. coli* expressing an Mtr respiratory pathway (versus the non-electrogenic *E. coli* strain). The necessity of this correlation is further evidenced by the fact that the reduction in polarizability of MtrABC-deficient *S. oneidensis* can be complemented by
providing a complete EET pathway in trans. In addition to removing or replacing EET components, increasing their redox activity by switching the growth conditions (e.g. respiration on an MFC anode or Fe(III) citrate versus fumarate) boosts cell surface polarizability. Moreover, EET components bearing different physiological functions (e.g. OmcB versus OmcZ in *G. sulfurreducens*, and MtrABC versus MtrDEF in *S. oneidensis*) lead to diverse effects on cell surface polarizability.

This study introduces surface polarizability as a novel physical property for assessing EET capacity. We show that surface polarizability can be measured using 3DiDEP noninvasively with low sample volume (~100 µL). We also show that this technique is sensitive enough to distinguish microbes that differ in the expression and redox activity of EET pathways. Our results suggest exciting potential for phenotypic-based screening of electrochemically active organisms using microfluidic dielectrophoresis. Compared to conventional screening methods, such as fluorescence-activated cell sorting (requiring specific fluorophore targets) and proteomic analysis (invasive and time-consuming), dielectrophoretic screening of cell surface polarizability may unlock a vast repertoire of EET-related biochemical applications. Examples include sorting a library of genetically engineered microbes for optimized MFC performance or iron reduction in the iterative process of directed evolution. In addition to EET, other surface features such as the presence of LPS or ion channels may also correlate with cell envelope polarizability. The structure of LPS has a significant impact on bacterial antibiotic resistance, while ion channels are crucial for regulation of membrane potentials and cell electrical signaling. This method will be useful as guidance for further dielectrophoresis-based phenotypic analysis of a diverse array of cells and organisms.
Chapter 4

3DiDEP Trapping Voltage Quantifies Microbial Lipopolysaccharide Composition

4.1 Introduction

This chapter demonstrates the application of 3DiDEP towards another interesting bacterial envelope phenotype – lipopolysaccharide (LPS) biosynthesis. The outer membrane (OM) enclosing Gram-negative bacteria is an asymmetric bilayer, in which phospholipids are localized in the inner layer, while LPS is the major component of the outer layer (Figure 4.1). [1, 109] The LPS molecule can be subdivided into three regions: (1) a hydrophobic membrane-anchor known as lipid A, (2) a core oligosaccharide, and (3) a distal polysaccharide known as O-antigen. [109]

4.1.1 Structure of LPS

Lipid A is composed of a backbone of two glucosamine (GlcN) residues linked (β-1,6) and esterified via the hydroxyl groups to fatty acids (Figure 4.1). [110, 111] The fatty acids attached to the glucosamine anchor the LPS into the outer leaflet of the bacterial OM through electrostatic and mainly hydrophobic interactions. The glucosamine disaccharide backbone is mostly phosphorylated. There are approximately $10^6$ lipid A residues, $10^7$ phospholipids and $10^5$ undecaprenyl phosphate-sugar molecules in an E. coli cell. [112, 113] Structures of lipid A can vary widely among different bacterial species. The potential differences refer to the structure of sugar moieties of the saccharide backbone, the number, position and length of the esterified acyl chains, the presence of charged groups on the polar heads, as well as the number of phosphate groups linked to the disaccharide backbone. [114] Sometimes more than one different lipid A structure can be found within a single species. [115]
Figure 4.1 Structure of the LPS in *E. coli* strains with an R1-type core. (top) A schematic showing the asymmetric structure of a Gram-negative bacterial envelope. (Bottom) A diagram depicting the chemical structure of the LPS molecule. Core residues are designated by sugar abbreviation and number. P, phosphate.
The core oligosaccharide is connected to lipid A via 3-deoxy-D-\textit{manno}-octulosonic acid (Kdo) (Figure 4.1). [114] The core oligosaccharide typically contains an inner region consisting of Kdo, heptose (Hep), ethanolamine, and phosphate, and an outer region that consists of hexoses. [110] The outmost polysaccharide region connected to the core, known as O-antigen, is made up of repeating units of four to six sugars that vary considerably in composition among different strains of bacteria.

4.1.2 Functions of LPS

The structural features of LPS are important for maintaining the selective permeability barrier function of the OM. LPS also plays an important role in maintaining the integrity of the bacterial OM by interacting with outer membrane proteins and divalent cations. In most cases, the lipid A and Kdo domains of LPS are required for cell growth. [112, 116] Other core domains and O-antigen sugars present in LPS are not needed for growth but protect bacteria from antibiotics and complement-mediated lysis. Moreover, LPS is one of the most conserved structures within all Gram-negative bacterial species, which makes LPS crucial for pathogen associated molecular pattern (PAMP) to be recognized by the host innate immune system. [111] LPS is recognized by Toll-like receptor 4 (TLR4) and MD-2 on host innate immune cells, which consequently leads to the production of pro-inflammatory cytokines that initiate the immune response. [117] The lipid A domain has received intensive attention, because it is a strong agonist of TLR4 signaling. Many pathogenic bacteria have been reported to modify their LPS or lipid A molecules in ways that significantly affect TLR4 signaling. [22-24] The core and O-antigen domains are important determinant for virulence, and consequently are present in most clinical and environmental isolates. [116] The O-antigen region in LPS is highly variable and antigenic, whereas the inner core region is conserved across genera, and thus attracting extensive investigations on the possibility of targeting the core oligosaccharide for general therapeutic application against Gram-negative bacterial infection. [118-121]
4.2 Conventional methods for LPS quantification

Conventional methods to characterize LPS often require large-scale isolation procedures and chemical methods, ranging from compositional analysis to sophisticated approaches involving various types of spectroscopy and nuclear magnetic resonance. Classically, LPS is extracted and purified from cell lysates, separated by electrophoresis in polyacrylamide gels, and then labeled with silver-staining, zinc sulphate, or fluorescent dyes. [122] However, approaches based on gel electrophoresis do not provide quantitative characterization of LPS. Lee et al. reported a Purpald assay to estimate LPS concentration by quantifying Kdo, which is the unique and conserved sugar component in LPS. [123] However, the Purpald method does not detect structural variations in other core regions of the LPS molecule. Mass spectrometry, such as electrospray ionization mass spectrometry (EI-MS) and matrix-assisted laser desorption ionization (MALDI)-mass spectrometry, has been utilized to quantify LPS by their mass-to-charge ratio. [124] However, these methods require specific instruments and trained personnel. Additionally, LPS extraction and purification are the prerequisite steps for most LPS characterization approaches, but the whole extraction process is time-consuming, involves the use of highly hazardous reagents (e.g. phenol/chloroform), and requires large sample volumes. Moreover, the requirement for hydrolysis of LPS into the lipid A and polysaccharide components is a potential cause of the loss and modification of the LPS components, leading to inaccurate estimation of the native LPS structures. Larrouy-Maumus et al. detected the lipid A in intact Gram-negative bacteria using MALDI-TOF mass spectrometry. However, the cells used in their study are heat inactivated, and this method has not been extended to characterize core oligosaccharide. The 3DiDEP microfluidic technique can be a powerful tool to surmount these challenges, and achieve rapid quantification of LPS structural variations in living bacteria without any chemical treatment or purification.

4.3 Quantification of E. coli LPS using 3DiDEP

In order to examine the sensitivity of the 3DiDEP platform for analyzing LPS structural differences, E. coli strains with diverse chemical structures were evaluated using the 3DiDEP microfluidic device.
4.3.1 *E. coli* LPS mutant strains

The studied *E. coli* strains, provided by Professor Christopher S. Hayes at UCSB, and their genotypes are tabulated in Table 4.1. The strain W3110 carries the plasmid pMF19 [125], which results in the formation of O-antigen at the end of the LPS molecules. The parent strain is derived from *E. coli* EPI100, with an additional gene deletion of *wzb*, which prevents the release of capsule polysaccharide. Mutant strains Δ*waac/F/P* are derived from the parent strain, with various portions of the LPS structure truncated. The heptosyltransferases WaaC and WaaF are glycosyltransferases involved in the synthesis of the inner core region of LPS. WaaC catalyzes the addition of the first heptose molecule to one Kdo residue of the Kdo2-Lipid A molecule. The mutant strain lacking WaaC (Δ*waac*) has the most minimal core, with only two Kdo residues. [114] Cells lacking the WaaF (Δ*waaf*) only differ from strain Δ*waac* by the addition of a single phosphorylated heptose (HepI) onto the first Kdo sugar. The LPS kinase WaaP is required for phosphate addition to HepI, and this reaction was found to be a prerequisite for the addition of the HepII residue, which in turn is required for the addition of a second phosphate at HepII. [126] Given this sequential action of the inner core biosynthesis process, deletion of WaaP alone is sufficient to eliminate all phosphate from the heptose region of the LPS inner core, resulting in a strain with only HepI and HepII sugars in its inner core (Δ*waaP*). [126] Additionally, a complemented version for strain Δ*waac* (pCH450-*waac*), and a strain carrying only an empty vector pCH450 serving as the control, were examined. The strain pCH450-*waac* was designed for a dynamic control of its LPS expression. Expression of WaaC is suppressed when the cells are maintained with glucose. Removal of glucose in their growth media induces expression of WaaC.

4.3.2 Growth conditions of *E. coli* LPS mutants

*E. coli* LPS mutant strains, inoculated from frozen stocks, were grown overnight in LB medium at 37 °C, shaking at 250 rpm. Spectinomycin (Spec) was supplied at 100 µg/mL for the growth of strain W3110 to maintain the plasmid pMF19. Streptomycin was supplied at 50 µg/mL for the growth of the parent strain. All the LPS mutant strains derived from the parent strain were grown with both Streptomycin (Strep) and Kanamycin (Kan) at 50 µg/mL. Strains carrying the plasmid pCH450 were also grown with tetracycline (Tet) at 20 µg/mL, kept away from light to mitigate the degradation of tetracycline. For strain W3110, the parent strain, and its LPS mutant strains
ΔwaaC/F/P, overnight cell cultures were back diluted 1:100, and handled following the method described in Section 2.2.1 (II) when reaching the stationary phase for 3DiDEP analysis. To achieve a dynamic control of WaaC expression in strain pCH450-waaC, both strain pCH450 (control for the plasmid burden) and strain pCH450-waaC were grown overnight in the LB-Strep-Kan-Tet medium with 0.4% glucose. Then, strain pCH450-waaC was centrifuged at 4,000 rpm for 4 minutes and resuspended in the LB-Strep-Kan-Tet medium with and without glucose with an initial OD600 of 0.1. The strain pCH450 was pelleted down and resuspended in the LB-Strep-Kan-Tet medium with glucose following the same procedure. Cells reaching mid-log phase were handled using the method described in Section 2.2.1 (II) for 3DiDEP analysis.

Table 4.1 E. coli LPS mutant strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relative genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>W3110</td>
<td><em>E. coli</em> W3110 strain with pMF19, O-antigen expressing strain.</td>
<td>Feldman et al. (1999) [125]</td>
</tr>
<tr>
<td>Parent</td>
<td>EPI100 with an additional gene deletion of <em>wzb</em></td>
<td>Constructed in the Hayes lab at UCSB.</td>
</tr>
<tr>
<td>ΔwaaC</td>
<td>The parent strain without <em>waaC</em></td>
<td></td>
</tr>
<tr>
<td>ΔwaaF</td>
<td>The parent strain without <em>waaF</em></td>
<td></td>
</tr>
<tr>
<td>ΔwaaP</td>
<td>The parent strain without <em>waaP</em></td>
<td></td>
</tr>
<tr>
<td>pCH450</td>
<td>Strain ΔwaaC carrying pCH450 empty vector to be used as a control for plasmid burden.</td>
<td></td>
</tr>
<tr>
<td>pCH450-waaC</td>
<td>Strain ΔwaaC carrying pCH450-waaC to be used for complementation of the WaaC-deletion strain.</td>
<td></td>
</tr>
</tbody>
</table>

4.3.3 3DiDEP trapping voltage is linearly correlated with *E. coli* LPS length

We examined the *E. coli* LPS mutant strains using 3DiDEP, and observed an inverse correlation between 3DiDEP trapping voltage and the length of expressed LPS molecules (Figure 4.2). The measured trapping voltage values are ranked in an ascending order as W3110 < Parent strain ≈ ΔwaaP < ΔwaaF < ΔwaaC (p < 0.05). Strain ΔwaaC, with the most minimal core oligosaccharide, appears to require the highest trapping voltage for 3DiDEP immobilization. Cells lacking WaaF differs from WaaC mutants by the addition of a single phosphorylated heptose onto the KdoI sugar (Figure 4.2). Comparison between strains ΔwaaC and ΔwaaF suggests that the 3DiDEP technique is sensitive enough to detect a single heptosylation event. Strain ΔwaaP, which has an additional
Figure 4.2 **3DiDEP trapping voltage is linearly correlated with the length of expressed *E. coli* LPS molecules.** The trapping voltages, measured using the 3DiDEP microfluidic device, for *E. coli* strain W3110, the parent strain, and its three LPS mutant strains lacking WaaC/F/P, are plotted in white bars (mean +/- std.). The corresponding chemical structures of the expressed LPS molecules are depicted above the bars. Gray bars show the trapping voltage measured for the WaaC-deletion strain complemented with an empty vector (control for the plasmid burden) and the strain complemented with WaaC in its suppression (glucose+) and induction (glucose-) states, respectively. Bold letters in the bar plot show the results from multiple comparison of group means using one-way ANOVA. Significant difference ($p < 0.05$) was found between groups not sharing a letter.
heptose unit but lacking the HepI phosphate in its inner core compare to strain \( \Delta\text{waaF} \), requires a further lower trapping voltage for 3DiDEP cell immobilization. Compared to the parent strain, which has the full-length LPS, strain W3110 expresses longer LPS with distal O-antigen repeats, and correspondingly requires a 0.5x lower trapping voltage.

To further verify that 3DiDEP trapping voltage can be used to quantify LPS compositional variations, a dynamic control of LPS expression was performed, using a complementary version (strain pCH450-\textit{waaC} ) of the WaaC-deletion mutant ( \( \Delta\text{waaC} \)). The expression of heptosyltransferases WaaC in strain pCH450-\textit{waaC} is suppressed when the cells are grown with glucose (glucose + in Figure 4.2), leading to the same LPS phenotype as that of strain \( \Delta\text{waaC} \). Removal of glucose in the growth medium (glucose - in Figure 4.2) induces WaaC expression, restoring the full-length LPS phenotype in strain pCH450-\textit{waaC}. As shown in Figure 4.2, the strain only complemented with the empty vector (strain pCH450) show a comparable trapping voltage compared to strain \( \Delta\text{waaC} \), suggesting that the introduction of pCH450 does not significantly influence the cell 3DiDEP performance. Comparing the trapping voltages required for strain pCH450-\textit{waaC} in its WaaC-suppression state (glucose +) and strain pCH450 further proves that cells with shortened LPS require a strong trapping voltage for 3DiDEP immobilization. Comparing the WaaC-suppression (glucose +) and induction (glucose -) states of strain pCH450-\textit{waaC} indicates that restoring the full-length of LPS reverses the increase in required trapping voltage.

### 4.4 Summary

This chapter demonstrate the first on-chip quantification of LPS compositional variations in living bacteria. A strong correlation between 3DiDEP trapping voltage and the length of \textit{E. coli} LPS has been illustrated. \textit{E. coli} mutants with a longer oligosaccharide truncated appear to require a higher trapping voltage. \textit{E. coli} lacking heptose in the LPS display a ‘deep rough’ phenotype, which has been characterized by hypersensitivity to novobiocin, detergents and bile salts. [127] Correspondingly, the studied ‘deep rough’ mutant, strain \( \Delta\text{waaC} \), requires the highest trapping voltage. Additionally, phosphate groups covalently attached to the heptose residues in the inner core interact with outer-membrane proteins as well as divalent cations, and are important to
stabilize the bacterial OM. Dephosphorylation of LPS inner core (strain ΔwaaP) also leads to a higher trapping voltage compared to the that of the parent strain. Our results suggest that bacterial LPS compositional diversity may contribute to the cell surface polarizability. The results also show the potential of using 3DiDEP to selectively enrich bacteria with different LPS structures, suggesting applications in rapid diagnostics and therapeutics.
Chapter 5

Nonlinear Electrokinetic Effects in Insulator-Based Dielectrophoretic Systems

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5.1 Introduction

The preceding chapters have shown the exciting promise of insulator-based dielectrophoresis (iDEP) for the characterization of bacterial envelopes. Recent advances in microfabrication [128] enables a broader application of iDEP microfluidic systems, including particle separation [129-135] and concentration [64, 134-137]. The use of geometric alterations to microfluidic confinements to create strong electric field gradients [138-140] forms the basis for iDEP. [64, 137, 140-146] In many iDEP-based operations, (including studies presented in the preceding chapters), no other nonlinear electrokinetic effects were assumed except for dielectrophoresis. However, this is only true for low external electric fields and low ionic strengths of the background medium. It should be noted that dielectrophoresis isn’t always the only significant electrokinetic phenomena. In many cases, especially with AC electric fields, fluid flow can be generated by two nonlinear electrokinetic phenomena: induced charge electroosmosis (ICEO) [147, 148] and electrothermal [149-153] flow.

ICEO is a result of the charges induced on the surface of a polarizable object exposed to an external electric field. Although microfluidic channels are made out of dielectric materials (of low but finite permittivity and conductivity) like polydimethylsiloxane (PDMS) [128] and poly(methyl methacrylate) (PMMA) [140, 141], strong electric fields can create significant ICEO near a
Electrothermal flow is generated by gradients in the fluid permittivity and conductivity resulting from Joule heating effects. Due to Gauss’ law, permittivity gradients result in local accumulation of charge density. The electric field interacts with these local charge densities and results in electrothermal flow. Electrothermal and ICEO effects, generating microvortices near microchannel constrictions, have been studied and utilized for applications including mixing [156] and concentration [157]. However, they can be undesirable in some situations (e.g. cell characterization and separation), and are often overlooked in the development of iDEP systems, which affects the accuracy and efficiency of particle manipulation.

Electrothermal flow in microfluidic devices has received much attention in the past few years [153, 158-160] because Joule heating is almost inevitable in electrokinetic devices. ICEO has received widespread theoretical attention for conducting and Janus particles [161-164], but ICEO over dielectric surfaces is relatively unexplored with a few exceptions. Pascall and Squires [165] studied ICEO over dielectric surfaces due to surface contamination. Zehavi and Yossifon [166] performed both theoretical and experimental investigations on the combined influence of ICEO, electroosmosis, electrophoresis and DEP at insulating microchannel junctions. More recently, Zehavi et al. [167] performed an experimental study of AC ICEO flows near a microchannel, by incorporating Joule heating effects at large conductivities. The work hints to the importance of the interplay between these two nonlinear electrokinetic effects in iDEP devices. Nevertheless, these behaviors are not fully understood. Previous studies on ICEO and/or electrothermal effects in insulator-based microsystems have shown microvortices with a size ~ 10 µm close to a dielectric corner. [153, 166] Lu et al. [168] demonstrated centimeter scale long-range vortices caused by a pair of co-planer electrodes. In this chapter, we report on the presence of millimeter scale fluid vortices located far from a dielectric constriction (where the local electric field is weak, see Figure 5.4) and adjacent to counter-rotating small vortices (scale to those observed by Kale et al. [153]) induced by AC electric fields. Previous studies on ICEO flows do not take into account the effects of constriction geometry variation, an important design parameter for iDEP devices. Kale et al. discussed the effect of constriction width on the flow field caused by a DC biased AC electric field. [153] However, Kale’s work did not consider the presence of ICEO phenomena. Additionally, since linear electrokinetics is comparable to the nonlinear electrokinetics in Kale’s work, the
electrothermal effect of reducing constriction width is partially diminished by the strong convective flow caused by the enhanced electroosmosis. This does not apply to purely nonlinear electrokinetic fluid flows.

The objective of this chapter is to demonstrate, from an experimental and theoretical viewpoint, the interplay of electrothermal and ICEO flows near microchannel constrictions with various geometries and fluid ionic strengths, which are crucial design factors for iDEP systems. Temperature rise and fluid velocities in 2D Gaussian-shaped constrictions were studied experimentally with supporting analytical estimations and numerical simulations. Additionally, we show qualitatively distinct recirculating flow patterns in 2D and 3D microchannel constrictions used for iDEP systems. Approximate analytical expressions for electrothermal and ICEO velocity scales are provided as a function of constriction geometry, bulk electrolyte concentration, and the applied electric field. Insights from this study will be useful in designing microfluidic systems for electrokinetic particle manipulation.

5.2 Microfluidic iDEP devices designed for the evaluation of electrothermal and ICEO effects.

Experiments were performed using both 2D (Figure 5.1a) and 3D (Figure 5.1b) iDEP microchannels. The 2DiDEP microchannels are 1 cm long and 50 µm deep, fabricated with PDMS (for both the ceiling and floor of the microchannel) using standard soft lithography (Figure 5.1a). [128] The PDMS layers on the top and bottom of the microchannel are ca. 5 mm thick, and the PDMS was bonded to a 1 mm thick glass slide by oxygen plasma treatment. The 2DiDEP microchannel wall profiles are defined by Gaussian functions, resulting in a channel width, \( W(x) \), varying with the axial coordinate, \( x \), as (Figure 5.1a):

\[
W(x) = W_0 \left[ 1 - (1 - \beta) \exp \left( -\frac{x^2}{2\sigma^2} \right) \right]
\]  

(5-1)
Figure 5.1 **Design of the 2D and 3D microchannels used for evaluating electrothermal and ICEO effects.** (a) A schematic of the experimental setup (side view), and a micrograph (top view) depicting the geometry of the 2DiDEP microchannel with a Gaussian-shaped constriction ($\beta = 1/10$) built in PDMS. The channel width, $W(x)$, is defined by Equation 5-1. (b) A schematic of the experimental setup (side view), and a micrograph (top view) depicting the geometry of the 3DiDEP microchannel with a $\beta = 1/100$ constriction built in PMMA. Scale bars: 100 µm.
where \( W_0 = 400 \, \mu m \) is the channel width far from the constriction, \( \beta \) is the constriction ratio defined as the ratio of the minimum to maximum channel cross-section area, and \( \sigma \) is the constriction span determined by the standard deviation of the Gaussian function. The 2D experiments were performed using microchannels with different constriction ratios (\( \beta = 1/10 \) and \( 1/20 \)), constriction spans (\( \sigma = 20 \) and \( 50 \, \mu m \)), and using potassium chloride with varying ionic strength (1 mM and 10 mM) as the experimental buffer. The 3DiDEP microchannels were fabricated on 3.1 mm thick PMMA by CNC micromilling (Figure 5.1b), following the same method described in Section 2.2.1. The PMMA microchannel cross-sectional area is 500 by 500 \( \mu m \) in the opening region, and 50 by 50 \( \mu m \) in the constriction region, generating a \( \beta = 1/100 \) constriction ratio (Figure 5.1b).

### 5.3 Scaling and analytical approximation

This section demonstrates approximate analytical expressions developed via scaling arguments to estimate the ICEO and electrothermal velocities near a microfluidic constriction whose width \( W(x) \) (and depth \( D \) in the case of a 3D constriction) vary as a function of the axial coordinate \( x \) from a maximum value of \( W_0 \).

#### 5.3.1 ICEO velocity

The differential equation governing the fluid potential, \( \phi_f \), is given as

\[
\nabla \cdot (\sigma_f \nabla \phi_f) = 0, \quad (5-2)
\]

where the fluid conductivity \( \sigma_f \) is temperature-dependent and hence can vary along the channel length. The Laplace equation governing the wall potential, \( \phi_w \), is given as

\[
\nabla^2 \phi_w = 0 \quad (5-3)
\]

For ICEO analysis, we use the complex amplitudes of the variables, i.e., \( \chi(t) = \text{Re}(\chi e^{i\omega t}) \) for variables \( \chi = \tilde{\phi}_w, \tilde{\phi}_f, E \), where the tilde indicates dimensionless variables. The Robin boundary
condition [154, 155, 169] relating the dimensionless fluid potential $\ddot{\phi}_f$ and the dimensionless wall potential $\ddot{\phi}_w$ is given as

$$\ddot{\phi}_w + \tilde{K}_{ICEO} \nabla \ddot{\phi}_w \cdot \hat{n} = \ddot{\phi}_f,$$  

(5-4)

where $\ddot{\phi}_w = \phi_w / (E_0 W_0)$, $\ddot{\phi}_f = \phi_f / (E_0 W_0)$, and $E_0$ is the electric field in the channel section with uniform cross-sectional area (far from the constriction).

$$\tilde{K}_{ICEO} = \tilde{\lambda}_D \tilde{\epsilon}_D \frac{i \tilde{\omega} \tilde{r}_r + 1}{i \tilde{\omega} \tilde{r}_r (i \tilde{\omega} + 1)^{\gamma/2}}$$

(5-5)

is the dimensionless parameter that governs the induced zeta potential magnitude and hence the ICEO flow profile. [154] $\tilde{r}_D = \epsilon_w / \epsilon_f$ is the wall to fluid permittivity ratio and $\tilde{r}_r = t_w / t_f$ is the wall to fluid dielectric relaxation time scale ratio. $t_w = \epsilon_w / \sigma_w$ is the wall dielectric relaxation time scale and $t_f = \epsilon_f / \sigma_f$ is the fluid dielectric relaxation time scale, where $\sigma_w$ and $\sigma_f$ are the wall and fluid conductivity, respectively. For a given bulk electrolyte concentration $c_{bulk}$, $t_f$ is also equal to the characteristic diffusion time scale $\lambda_D^2 / D_{ion}$ across the electric double layer of thickness $\lambda_D = \sqrt{\epsilon_f k_B T / 2 z^2 e^2 c_{bulk}}$, where $D_{ion}$ is the ionic diffusivity. $\tilde{\lambda}_D$ is the dimensionless Debye length given by $\tilde{\lambda}_D = \lambda_D / W_0$ and $\tilde{\omega} = \omega / (t_f^{-1})$ is the dimensionless applied electric field frequency. The divergence theorem dictates that the x-component of electric field $E = E_x i + E_y j$ satisfies

$$\tilde{E}_x = \frac{E_x}{E_0} \sim \frac{A_0}{A_e(x)}.$$  

Here, $A_0$ is the maximum cross-sectional area of the channel, and $A_e(x)$ is the cross-sectional area at the axial location $x$. The temperature dependence on fluid conductivity $\sigma_f$ is not taken into consideration when approximating the ICEO velocity. This will be discussed further in the Section 5.5.2. The dimensionless induced zeta potential is given by

$$\tilde{\zeta}_x = \tilde{\phi}_w - \tilde{\phi}_f = -\left(\nabla \tilde{\phi}_w \cdot \hat{n}\right) \tilde{K}_{ICEO} \sim -\left(\nabla \tilde{\phi}_f \cdot \hat{n}\right) \tilde{K}_{ICEO} = \left(\tilde{E}_x n_x + \tilde{E}_y n_y\right) \tilde{K}_{ICEO}$$

(5-6)

(For dielectric walls with small induced zeta potentials, the wall potential is also approximately of the same order as the fluid potential). Here $n_x$ and $n_y$ are the x and y components, respectively, of
the local normal vector given by \( \hat{n} = \left[ -f'(x) / \sqrt{1 + f'(x)^2} \right] e_x + \left[ 1 / \sqrt{1 + f'(x)^2} \right] e_y \) for a smooth, continuous wall profile \( f(x) \). Therefore, given \( \tilde{E}_y \ll \tilde{E}_x \),

\[
\xi_i \sim \tilde{K}_{ICEO} \tilde{E}_x n_x \sim -\tilde{K}_{ICEO} \frac{A_0}{A_x(x)} \frac{f'(x)}{\sqrt{1 + f'(x)^2}}.
\]

Hence the \( x \)-component of the ICEO velocity scale is given by

\[
u_{ICEO} \sim -\frac{\epsilon f_{\xi_i} E_x}{\eta} \sim \frac{\epsilon f_{\xi_i} E^2 W_0 \tilde{K}_{ICEO}}{A_0 A_x(x)} \frac{f'(x)}{\sqrt{1 + f'(x)^2}},
\]

where \( \eta \) is the fluid viscosity.

### 5.3.2 Temperature and electrothermal velocity

To model electrothermal flow, we solve the time-averaged Stokes equation given as

\[
-\nabla p + \eta \nabla^2 \mathbf{u} + \langle f_{ET} \rangle = 0,
\]

where \( \langle f_{ET} \rangle = \frac{1}{2} \text{Re} \left[ \frac{\epsilon_f \alpha - \gamma}{1 + i \omega t_f} \left( \nabla T \cdot \mathbf{E} \right) \overline{\mathbf{E}} \right] - \frac{\epsilon_{fo}}{4} \alpha \left( \mathbf{E} \cdot \mathbf{E} \right) \nabla T \) (overbar denotes complex conjugate) is the electrothermal body force term. Here, \( \alpha \) is the temperature coefficient of the fluid permittivity, i.e., \( \tilde{\epsilon}_f = \epsilon_f / \epsilon_{fo} = 1 + \alpha(T - T_0) \), and \( \gamma \) is the temperature coefficient of the fluid conductivity, i.e., \( \tilde{\sigma}_f = \sigma_f / \sigma_{fo} = 1 + \gamma(T - T_0) \). \( T \) is the temperature whose distribution is modeled by the heat equation, given as

\[
\rho_f C_p \left( \mathbf{u} \cdot \nabla T \right) = \nabla \cdot \left( k \nabla T \right) + \langle \sigma_f \mathbf{E} \cdot \overline{\mathbf{E}} \rangle.
\]

Here \( \langle \sigma_f \mathbf{E} \cdot \overline{\mathbf{E}} \rangle \) denotes the time-averaged Joule heating source term, \( \rho_f \) is the fluid density, \( C_p \) is the fluid heat capacity, and \( k \) is the fluid thermal conductivity.
In many iDEP operational conditions, including the experimental conditions used in this chapter, the Reynolds number is \( \text{Re} \leq 0.01 \) and the Prandtl number is \( \text{Pr} < 10 \), leading to a Peclet number, \( \text{Pe} = \text{RePr} \) less than 0.1. Thus, the convection terms in Equation 5-10 are negligible compared to the conduction and source terms. Since the Biot number along channel depth and transverse direction is much lower than the Biot number along the axial direction, for scaling one can assume 1D temperature variation along the channel axial direction within the fluid domain. It is noteworthy that the reported heat transfer coefficient found in the literature can vary from 10 to 10³ W/(m²·K).

For instance, Prabhakaran et al. [170] estimated the heat transfer coefficient \( H_T = 6 \) W/(m²·K), while Sridharan et al. [171] used a heat transfer coefficient \( H_T = 20,000 \) W/(m²·K). The Fourier number for the fluid domain is high (\( \text{Fo} \sim 10^4 \) with a time \( t = 1 \) min and a characteristic length \( D/2 = 25 \mu\text{m} \)), indicating fast heat diffusion compared to heat storage within the fluid body. However, the Fourier number for the PDMS domain is much lower (\( \text{Fo} \sim 0.25 \) with a time \( t = 1 \) min and a characteristic length of 5 mm) than that of the fluid, suggesting time-dependent heat conduction within the PDMS domain and quasi-steady state in the fluid domain. In many iDEP systems the electric fields are applied for just a few minutes in order to diminish the thermal effects on biological samples. [140, 141, 143-145] For these cases, we consider heat conduction in PDMS as conduction in a semi-infinite body, and estimate transient temperature at the center of the constriction and regions far from the constriction (solid lines in Figure 5.7b) using Carslaw’s and Jaeger’s solution (see Appendix A-2 for detailed expressions) [172]. For the steady-state case (where electric fields are applied for \( \sim 10 \) min or longer), the temperature distribution can be approximated by a 1D fin-like equation with a Joule-heating source term given as

\[
\frac{d}{d\tilde{x}} \left[ A_x(\tilde{x}) \frac{dT_{\text{avg}}}{d\tilde{x}} \right] + \frac{1}{2} \frac{\sigma \rho_0^2 E_0^2}{k T_0} A_x(\tilde{x}) \tilde{E}_x^2 \frac{H_T P W_0^2}{k} \left( \tilde{T}_{\text{avg}} - 1 \right) = 0 \tag{5-11}
\]

where \( \tilde{T}_{\text{avg}} \) denotes the cross-section averaged dimensionless temperature given by \( \tilde{T}_{\text{avg}} = T_{\text{avg}} / T_0 \), and \( P \) is the perimeter over which heat transfer occurs. Note that we have included only the \( x \)-component of the electric field in the source term, as the \( y \)-component of the electric field is small in comparison. To get an approximate scaling formula for electrothermal velocity, we use the expression suggested by Gonzalez et al. [152] for flow around electrodes.

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\[ u_{ET,2d} = \xi_{2d} 0.0538 \frac{f_0 E_0^2 W^2}{2\pi^2 \eta} \gamma \left( \frac{\Delta T_{avg}}{L_c} \right), \quad u_{ET,3d} = \xi_{3d} 0.234 \frac{f_0 E_0^2 W^2}{2\pi^2 \eta} \gamma \left( \frac{\Delta T_{avg}}{L_c} \right) \] (5-12)

Here the geometric factors \( \xi_{2d,3d} \) are O(1), and are obtained by comparing one analytical result with the COMSOL simulation. \( L_c \) is the constriction length (Figure 5.1). For 2D constrictions, \( L_c \) is estimated by six times the standard deviation (\( \sigma \)) of the Gaussian-shaped constriction. For 3D constrictions, \( L_c \) is 450 \( \mu m \).

### 5.4 Numerical simulation

We performed 3D time-dependent numerical simulations considering the combination of ICEO and electrothermal flows using COMSOL Multiphysics 5.1 (Burlington, MA), with the simulation parameters summarized in Table 5.1. The computational domain included both the fluid and microchannel wall domains (Figure 5.2). We solved Equations (5-2) and (5-3) for fluid and wall potential with boundary condition given by Equation (5-4) at the wall-fluid interface. At the interface between the wall and the ambient we used an insulating boundary condition, i.e., Neumann boundary condition on wall potential. To obtain the temperature profile we solved the energy equation given by Equation (5-10) in both the fluid and wall domains. The axial temperature gradient was assumed to be equal to zero on the end of either side of the microchannel (as shown in Figure 5.7c) because the temperature profile becomes constant due to a balance between axial conduction, Joule heating and heat loss through the channel walls. Natural convection at the wall-air interface was assumed to be very small compared to the conduction heat transfer from the fluid to wall domains. The fluid flow in the microchannel was solved by the Navier-Stokes equation given by Equation (5-9) with ICEO slip velocity boundary condition at the wall, given by Equation (5-8), to account for both ICEO and electrothermal effects. We assumed equal pressures at the microchannel inlet and outlet.
Figure 5.2 *Computational geometry used in the 3D transient numerical model for an iDEP microfluidic channel with a 2D Gaussian-shaped constriction.* Dimensions, governing equations for each computational domain, and boundary conditions (BC) are also labeled. The inset is a magnified view of the microchannel constriction.
Table 5.1 Parameters and material properties used for analytical approximation and COMSOL modeling. [153, 170]

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$E_0$</td>
<td>Applied electric field</td>
<td>100 V/cm, 400 V/cm for the 2D constriction</td>
</tr>
<tr>
<td></td>
<td></td>
<td>106 V/cm, 71 V/cm for the 3D constriction</td>
</tr>
<tr>
<td>$\omega$</td>
<td>Electric field frequency</td>
<td>1 kHz for 2D constrictions</td>
</tr>
<tr>
<td></td>
<td></td>
<td>25 kHz for 3D constrictions</td>
</tr>
<tr>
<td>$T_0$</td>
<td>Ambient temperature</td>
<td>293 K</td>
</tr>
<tr>
<td>$c_{bulk}$</td>
<td>Buffer concentration</td>
<td>1 mM, 10 mM</td>
</tr>
<tr>
<td>$D_{ion}$</td>
<td>Ionic diffusivity</td>
<td>$2 \times 10^{-9}$ m$^2$/s</td>
</tr>
<tr>
<td>$\varepsilon_{r0}$</td>
<td>Fluid relative permittivity at reference temperature</td>
<td>80</td>
</tr>
<tr>
<td>$\alpha$</td>
<td>Temperature coefficient of the fluid permittivity</td>
<td>-0.0046 1/K</td>
</tr>
<tr>
<td>$\gamma$</td>
<td>Temperature coefficient of the fluid conductivity</td>
<td>0.02 1/K</td>
</tr>
<tr>
<td>$k_{PDMS}$</td>
<td>Thermal conductivity of PDMS</td>
<td>0.16 W/(m·K)</td>
</tr>
<tr>
<td>$k_f$</td>
<td>Thermal conductivity of fluid</td>
<td>0.6 W/(m·K)</td>
</tr>
<tr>
<td>$\eta$</td>
<td>Fluid viscosity</td>
<td>$8.66 \times 10^{-4}$ Pa·s</td>
</tr>
<tr>
<td>$\rho_{PDMS}$</td>
<td>PDMS density</td>
<td>970 kg/m$^3$</td>
</tr>
<tr>
<td>$\rho_f$</td>
<td>Fluid density</td>
<td>1000 kg/m$^3$</td>
</tr>
<tr>
<td>$C_{p,PDMS}$</td>
<td>PDMS heat capacity</td>
<td>1460 J/(kg·K)</td>
</tr>
<tr>
<td>$C_{p,f}$</td>
<td>Fluid heat capacity</td>
<td>4186 J/(kg·K)</td>
</tr>
<tr>
<td>$\tilde{r}_f$</td>
<td>Wall to fluid dielectric relaxation time scale ratio</td>
<td>$1 \times 10^{10}$</td>
</tr>
<tr>
<td>$\tilde{r}_D$</td>
<td>Wall to fluid permittivity ratio</td>
<td>0.025</td>
</tr>
<tr>
<td>$H_T$</td>
<td>Natural convection coefficient to the ambient</td>
<td>0 W/(m$^2$·K) (heat transfer by natural convection is negligible compared to heat loss by conduction through the PDMS walls)</td>
</tr>
<tr>
<td>$H_{T,2D}$</td>
<td>Heat transfer coefficient in the 1D fin equation for the 2D constriction</td>
<td>800 W/(m$^2$·K)</td>
</tr>
<tr>
<td>$H_{T,3D}$</td>
<td>Heat transfer coefficient in the 1D fin equation for the 3D constriction</td>
<td>15,000 W/(m$^2$·K)</td>
</tr>
</tbody>
</table>
5.5 Nonlinear electrokinetics in 2DiDEP microchannels

5.5.1 Nonlinear electrokinetic flow velocity

We quantified the electrokinetic flow motion in 2DiDEP systems described in Section 5.2 induced by pure AC electric fields, in order to decouple the linear and nonlinear electrokinetic effects. The fluid motion in 2D microchannels (Figure 5.4) was observed by tracing 1 \( \mu m \) (diameter) fluorescent particles suspended in 1 mM and 10 mM potassium chloride. The 1 \( \mu m \) particles can be considered as negative tracers of the background fluid motion without exhibiting significant DEP forces because similar results were obtained using 200 nm particles (Figure 5.4). Particle trajectories and their velocities along pathlines were determined using ImageJ 1.51a with a 2D particle tracking algorithm [173]. Figure 5.4 shows the particle trajectories near Gaussian-shaped constrictions with various constriction ratios (\( \beta = 1/10 \) and \( 1/20 \)) and constriction spans (\( \sigma = 20 \) and \( 50 \) \( \mu m \)). The particles exhibit symmetric double-set vortex flow patterns. Figure 5.5a and 5.5b show particle velocities averaged over particle pathlines for the inner and outer vortices (locations indicated in Figure 5.5c). Figure 5.5c shows a measured velocity field obtained by processing the fluorescent images of microbead motion using a digital particle image velocimetry (PIV) MATLAB package [174]. The inner set of four vortex flows are located closer (ca. 15 \( \mu m \)) to the constriction, smaller (\( 10 - 100 \) \( \mu m \)) in size, and rotating faster (by an order of magnitude) in the opposite direction compared to that of the outer set (Figure 5.5). Increasing the electrolyte ionic strength by an order of magnitude enhances flow velocity by an order of magnitude (Figure 5.5a) as well, which is consistent with electrothermal flow behavior (Equation 5-11 and 5-12). The classical model predicts electrothermal flow increases linearly with the fourth power of applied electric field, i.e. \( u_{ET} \sim E_0^4 \). However, the best power-fit curves for inner vortex velocity versus applied electric field indicate a power ranging from 3.18 to 4.39. Moreover, significant differences were found (\( p < 0.005 \)) between inner vortexing velocities measured in 10 mM potassium chloride at an electric field higher than 250 V/cm in microchannels with different Gaussian geometries (Figure 5.5a). For example, the inner vortexing velocity measured in a \( \beta = 1/20 \) microchannel is ca. 1.4 times of that measured in a \( \beta = 1/10 \) microchannel with the same constriction span (\( \sigma \)).
Figure 5.4 Particle tracking indicates flow patterns in 2DiDEP systems. Representative fluorescent images indicating the vortical flow pattern near three different Gaussian-shaped (see Equation 5-1) 2D constrictions (differentiated by profile colors) at 1 kHz AC electric field with 300 V/cm RMS applied (tracer: 1 µm microspheres).

Figure 5.4 DEP effects are insignificant near 2DiDEP constrictions. Inner vortex velocities (mean +/- 95% CI) measured near $\beta = 1/20, \sigma = 50 \, \mu m$ constrictions in 1 mM potassium chloride buffer using microsphere tracers with diameters of 200 nm (blue triangles) and 1 µm (black circles) at various electric field intensities. Paired-sample t-test indicates no significant difference between the results obtained using different microspheres. Because the $\beta = 1/20$ constriction creates higher electric field gradient and thus induces stronger DEP (compared to the case of $\beta = 1/10$), and the DEP force is proportional to particle volume, this result suggests that 1 µm beads are sufficiently small as passive tracers of the fluid motions.
Figure 5.5 **Measured flow velocity distribution induced by nonlinear electrokinetic effects in 2DiDEP systems.** Velocity averaged over microsphere pathlines of the inner (a) and outer vortex (b) measured in potassium chloride buffer (1 and 10 mM) by a previously reported 2D particle tracking algorithm and ImageJ 1.51a (see Supporting Information) at various electric field intensities, and corresponding power-fit curves (colors for different channel geometries correspond to that in Figure 5.4). (c) Velocity field measured by Particle Image Velocimetry using PIVlab MATLAB program near a $\beta = 1/10$, $\sigma = 50 \mu$m constriction in 10 mM potassium chloride at 200 V/cm RMS electric field.
5.5.2 Temperature rise induced by Joule heating effects

For a detailed understanding of the electrothermal effects near the 2D Gaussian constrictions, we established a 3D numerical model, and it is seen to predict the measured temperature field with a good agreement (Figure 5.7a). The temperature distribution was determined by measuring the fluorescent intensity of 0.055 g/L Rhodamine B (a temperature-sensitive dye) in potassium chloride for various ionic strengths (1 and 10 mM) and applied electric fields. Fluorescent time lapse image sequences were recorded using a CoolSNAP HQ2 cooled CCD camera (Photometrics, Tucson, AZ) fitted to an inverted fluorescence microscope (Nikon, Tokyo, Japan) controlled by a Micro-Manager multi-acquisition package. The fluorescent intensity data was converted to temperature based on the third-order polynomial relation proposed by Ross et al. [175] as

\[
T = 132 - 250(C \cdot I) + 220(C \cdot I)^2 - 79(C \cdot I)^3
\]  

(5-13)

where \(T\) is the temperature (in degree Celsius), \(I\) is the fluorescent intensity normalized by its value at room temperature (20 °C), and \(C = I(T = 20°C)/I(T = 23°C) = 1.062\) is the correction factor to account for the fact that Ross et al. normalized fluorescence intensity to a reference temperature of 23 °C. The conversion equation (Equation 5-13) between fluorescence intensity and temperature was validated by heating the 0.055 g/L Rhodamine B solution in the PDMS fluidic reservoirs and measuring the temperature (Figure 5.7) with a Traceable Ultra Long-Stem Thermometer (Traceable Products, Control Company, Texas, USA).

We recorded the transient development of temperature rise at two locations in the microchannel, constriction center and regions far from the constriction. As illustrated in Figure 5.7b, our analytical approximation (Applendix A-2, Equation A5) compares well with the experimental data and numerical simulation. The AC electric field was applied at \(t = 5\) s, the temperature increased sharply within a few milliseconds, and the rate decays with time (Figure 5.7b). As predicted by our scaling analysis (see Section 5.3.2), heat conduction in the PDMS domain did not reach steady state within 1 min after the application of electric field. Figure 5.7c shows the temperature distribution along channel centerline measured at \(t = 50\) s, along with the COMSOL simulation (at
Temperature rise in narrower constrictions (\(b = 1/20\)) is more significant compared to that in the case of \(b = 1/10\) (Figure 5.7b). However, this difference can be overestimated if the temperature dependence of conductivity 
\[
\sigma_f = \sigma_{f0} \left[ 1 + \gamma (T - T_0) \right]
\]
is neglected. For instance, in a \(b = 1/10\) and \(s = 50\ \mu\text{m}\) constriction at a buffer concentration of 10 mM, the estimated peak temperature at 300 V/cm is 333 K if we assume a constant conductivity and 319.25 K if we assume a temperature-dependent conductivity. The latter is in closer agreement with experiments (Figure 5.7b and 5.7c). One of the reasons is that

\[
\frac{E_{\text{center}}}{E_0} \sim \frac{1}{\beta} \frac{\sigma_{f0}}{\sigma_{f,\text{center}}},
\]

where \(\frac{E_{\text{center}}}{E_0}\) and \(\frac{\sigma_{f,\text{center}}}{\sigma_{f0}}\) are the ratios of electric field and fluid conductivity at the constriction center versus regions far from the constriction, respectively. For a temperature rise of 30 K and a temperature coefficient of the fluid conductivity of \(\gamma = 0.02\ \text{1/K}\) \([153]\), \(\frac{\sigma_{f0}}{\sigma_{f,\text{center}}} = 62.5\%\), resulting in an overestimation of the maximum electric field by 37.5% using a constant fluid conductivity. Moreover, since the heat generation per unit length along the

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**Figure 5.6** Verification of the conversion equation of Rhodamine B fluorescent intensity into temperature. Measured temperature (in degree Celsius) versus fluorescent intensity normalized to the value at 20 °C (blue circles) and the best fit curve (red dashed line, \(R^2 = 0.99885\)) compare well to the third-order polynomial relation (Equation 5-13) by Ross et al. (gray solid line).
Figure 5.7 Temperature rise near 2DiDEP constrictions. (a) Comparison of the distribution of temperature rise (reference temperature: 293 K) near a $\beta = 1/20$, $\sigma = 50 \mu m$ constriction 50 sec after the application of electric field by COMSOL simulation (top) and experimental data measured using Rhodamine B (bottom). (b) Transient temperature rise at the constriction center (triangles) and far from the constriction (circles) measured using Rhodamine B, along with COMSOL simulation (dashed lines) and analytical approximation (solid lines) by Car-slaw’s solution (Appendix A-2, Equation A5) for the three different channel geometries. (c) Temperature distribution along channel centerline measured 50 sec after the application of electric field, along with COMSOL simulation (dashed lines) and analytical estimates (solid lines) for various constriction geometries. Experimental conditions: 10 mM potassium chloride, 1 kHz and 300 V/cm RMS electric field. Colors indicating channel geometries correspond to that in Figure 1.
microchannel is proportional to the channel width $W(x)$ at the constriction, for narrower constrictions (lower $\beta$), the decreased channel width partially compensates for the influence of the increased local electric field upon heat generation.

5.5.3 Comparison between ICEO and electrothermal effects in 2DiDEP systems

Comparing the numerically predicted pure ICEO (Figure 5.8a) and electrothermal flow field (Figure 5.8b) with the experimental data (Figure 5.4 and Figure 5.5c) suggests that the flow results predominantly from electrothermal effects at this condition. In the absence of electrothermal effects, the flow due solely to ICEO would lead to a single-set of vortices (Figure 5.8a) that have similar characteristics (flow direction and speed) as the observed outer vortex flow in the case of a 2D constriction (Figure 5.5c). Figure 5.9a-c compare the estimated ICEO and electrothermal velocities. The ICEO line intercepts with the electrothermal line for the case of 100 V/cm applied electric field at ca. 2 mM for the $\beta = 1/10$ and $\sigma = 50 \mu m$ constriction, 4 mM for the $\beta = 1/10$ and $\sigma = 20 \mu m$ constriction, and 6 mM for the $\beta = 1/20$ and $\sigma = 50 \mu m$ constriction. This suggests that for notable nonlinear electrokinetic flows (velocity higher than 1 $\mu m/s$) near a 2DiDEP constriction, electrothermal effects are always more significant compared to ICEO effects for a buffer concentration higher than the range of 1 - 10 mM. For lower buffer concentrations, the interplay between electrothermal and ICEO effects depends on both buffer concentration and applied electric field.

Comparing COMSOL simulations (Figure 5.8b) and the experimental PIV results (Figure 5.8c) of the velocity profiles in the channel transverse direction confirms the formation of the inner and outer vortices in 2D constrictions. The inner vortices have been reported in a number of studies [153, 171]. In contrast, the outer vortices are located hundreds of microns away from the constriction (where the electric field gradient is low), and the circulation extends to ~ 1 mm in distance. This millimeter-scale vortex has not been characterized in previous studies. Although Lu et al. have reported similar flow patterns near a pair of parallel electrodes in a microfluidic channel, the flow direction they found was opposite to that in this work, and the outer vortex in Lu’s work extends to several centimeters, which can be suppressed by reducing the characteristic length of
Figure 5.8 **ICEO and electrothermal flow.** (a-b) COMSOL simulations for the pure ICEO flow (a) and electrothermal flow patterns (b) in 10 mM potassium chloride near a $\beta=1/10$, $\sigma = 50 \mu m$ constriction at 1 kHz electric field frequency with 200 V/cm RMS applied. Electrothermal flow dominates over ICEO flow. (c) Comparison between velocity magnitudes along the channel width direction ($y$) at a distance $x = 30 \mu m$ (inner vortex) and $x = 150 \mu m$ (outer vortex) from the constriction center ($x = 0$) by COMSOL simulation (solid line, inner vortex; dashed line, outer vortex) and experiments (blue circles, inner vortex; red triangles, outer vortex). Experimental conditions correspond to those in the panel (b). (d) Y-distribution of electrothermal flow velocity at $x = 15 \mu m$ (solid lines) and $x = 150 \mu m$ (dashed lines) with 10 mM ion concentration at various applied electric fields (200, 250 and 300 V/cm RMS) by COMSOL simulation for the three constriction geometries.
Figure 5.9 **Comparison between electrothermal and ICEO flow in 2DiDEP systems.** Comparison between the analytical scaling formulae for electrothermal (ET) and ICEO velocities and the maximum velocity observed in the COMSOL simulation for 2D constrictions with various geometric parameters: (a) $\beta = 1/20, \sigma = 50 \mu m$; (b) $\beta = 1/10, \sigma = 20 \mu m$; and (c) $\beta = 1/10, \sigma = 50 \mu m$. The applied electric fields are 100 V/cm (low) and 400 V/cm (high). Color-maps of the ratio (in $\log_{10}$) of electrothermal and ICEO velocities in steady-state by analytical scaling for 2D constrictions with various geometric parameters: (d) $\beta = 1/20, \sigma = 50 \mu m$; (e) $\beta = 1/10, \sigma = 20 \mu m$; and (f) $\beta = 1/10, \sigma = 50 \mu m$. The velocities are of equal order of magnitude along the dashed line. The white region corresponds to a temperature rise of more than 80 K.
the microchannel [168]. Our numerical prediction of the velocity profile along the microchannel transverse direction for the inner vortices compares well with the experimental PIV data, but the velocity of outer vortices is underestimated (Figure 5.8c). One possible reason is that there can be other nonlinear electrokinetic effects present, which is beyond the scope of this study. For instance, both the temperature gradient and ICEO flows can facilitate the formation of bulk electrolyte concentration gradients near the constriction, which could result in higher velocities in the outer vortices. [176] The inner vortices display a dependence on the channel constriction ratio (Figure 5.8d). For instance, in the narrowest constriction ($\beta = 1/20$), the velocity reaches a minimum at the channel centerline, whereas in the case of $\beta = 1/10$, the velocity reaches its maximum at the channel centerline, suggesting opposite vorticity of the inner vortices in these two cases. However, the COMSOL simulation did not indicate significant differences in the outer vortices among microchannels with different geometries (Figure 5.8d).

### 5.6 Nonlinear electrokinetics in 3DiDEP microchannels

Particle trajectories are qualitatively very different near a 3D constriction (Figure 5.1b) [140] with a $\beta = 1/100$ constriction ratio, as suggested by our 3D transient numerical model considering the combination of ICEO and electrothermal effects (Figure 5.11a). The trajectories exhibit both field dependence (76 V RMS vs. 106 V RMS) as well as particle size (200 nm, 1 $\mu$m, and 6 $\mu$m) dependence (Figure 5.10). The trajectories exhibit a single set of vortices and are not confined to a single plane, i.e., there are additional velocity components in the channel depth direction (Figure 5.11a). Moreover, the trajectory varies with particle size. For instance, the 200 nm particle streaks are consistent with the numerically predicted streamlines (Figure 5.10b), suggesting that DEP is not significant compared to electrothermal and ICEO effects in this case. However, in the case of 6 $\mu$m beads, the particle recirculation plane is perpendicular to the focal plane of the microscope (Figure 5.10e and 5.10f). This particle size dependence indicates that the effects of DEP forces are no longer negligible compared to the hydrodynamic drag for 6 $\mu$m beads.
Figure 5.10 Particle trajectories near a 3DiDEP constriction ($\beta =1/100$) for various particle sizes (200 nm, 1 $\mu$m, and 6 $\mu$m) and applied RMS electric fields (71 V/cm and 106 V/cm). Panel (b) compares numerically predicted fluid streamlines projected on the top-view plane (top) compared to the experimentally obtained particle streak image (bottom). In each case the bulk electrolyte is 1 mM potassium sulfate and the electric field frequency is 25 kHz. All fluorescent images were taken with an exposure time of 10 seconds. Scale bar: 100 $\mu$m.
Figure 5.11 **Comparison of numerically predicted and measured nonlinear electrokinetic flow velocity in a 3DiDEP constriction.** (a) 3D streamlines induced by 106 V/cm RMS electric field predicted by the 3D transient numerical model considering the combination of ICEO and electrothermal effects. Line color indicates the magnitude of combined electrothermal and ICEO velocity. (b) Combined electrothermal and ICEO flow velocity field near the floor of the microchannel constriction comparing the numerical simulation obtained from the 3D transient model (left) with the experimental data obtained by tracing 1 µm microspheres (right). The color scale indicates the velocity magnitude, and arrows show the normalized velocity vectors. (c) Radial distribution of velocity measured (black circles) between an angle $\pi/12 < \theta < \pi/4$ from the channel centerline. Also illustrated is the numerical prediction of combined electrothermal and ICEO velocity versus the radial distance from the constriction center along $\theta = \pi/12$ (red solid line) and $\theta = \pi/4$ (blue dashed line), respectively. The experimental condition corresponds to Figure 5.10c.
Due to the additional velocity component in the channel depth direction, it is challenging to experimentally visualize and quantify the velocity field near the 3DiDEP constriction. We measured the 2D velocity field near the floor of the 3D microchannel constriction (where the velocity component in channel depth direction is small), and compared it with the numerical simulation (Figure 5.11b). Given that our analytical method estimates an electrothermal velocity of $306 \mu m/s$ (Figure 5.12a) and negligible ICEO velocity for the same condition, it is seen that both our analytical formula and numerical model predict the 3DiDEP experiment reasonably well. Figure 5.11c shows a radial velocity distribution (with the origin at the constriction center) measured within the sectors whose boundaries are $\theta = \pi/12$ and $\theta = \pi/4$ from channel centerline. The velocity magnitude increases and then drops exponentially along the radial direction, with a peak located $\sim 30 \mu m$ away from the constriction center. The simulation predicts a trend of the radial velocity distribution that is consistent with the experimental data, but the magnitudes were

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**Figure 5.12 Comparison between electrothermal and ICEO flow in 3D iDEP systems.** (a) Comparison between the analytical scaling formulae for electrothermal and ICEO velocities and the maximum velocity observed in the COMSOL simulation for 3D constrictions. The applied electric fields are 71 V/cm (low) and 106 V/cm (high). (b) Color-maps of the ratio (in $\log_{10}$) of electrothermal and ICEO velocities in steady-state by analytical scaling for 3D constrictions. The velocities are of equal order of magnitude along the dashed line. The white region corresponds to a temperature rise of more than 80 K.
underestimated (Figure 5.11c), suggesting that there can be other nonlinear electrokinetic effects (e.g. diffusiophoresis and dielectrophoresis) in play. The 3D COMSOL model suggests that the maximum velocities resulting from electrothermal and ICEO effects at 106 V RMS applied voltage are 1540 µm/s and 104 µm/s, showing that both effects are important in the 3D case, though electrothermal effects still dominate. Figure 5.12 shows that for notable nonlinear electrokinetic flows (velocity higher than 1 µm/s) near 3DiDEP constrictions (\(\beta = 1/100\)), electrothermal effects dominate over ICEO effects in the case of buffer concentrations higher than 1 mM.

As shown in Figure 5.9 and Figure 5.12, our analytical scaling expressions capture the ICEO and electrothermal velocity trends fairly accurately after using geometric factors which are \(O(1)\) (\(\xi_{2d,ICEO} = 0.22, \xi_{2d,Electrothermal} = 0.5\) for the \(\beta = 1/10\) constriction; \(\xi_{2d,ICEO} = 0.44, \xi_{2d,Electrothermal} = 0.18\) for the \(\beta = 1/20\) constriction; and \(\xi_{3d,ICEO} = 5, \xi_{3d,Electrothermal} = 0.8\) for the 3D constriction). Using these approximate scaling expressions, one may estimate the relative importance of ICEO and electrothermal flow effects in microfluidic constrictions over a wide range of experimental conditions.

Figure 5.13 Time estimated to reach boiling temperatures. The period of time for the fluid to reach boiling temperature (\(\Delta T = 80\) K) in 2DiDEP microchannel constrictions with various buffer concentrations, applied RMS voltages, and constriction ratios (\(\beta\)) estimated using the analytical formula (Equation A5).
parameters without resorting to computationally expensive numerical simulations. For instance, Figure 5.9d-f and Figure 5.12b present color-maps of the relative importance of these two effects in steady-state for 2D and 3D constrictions, respectively. The dashed line corresponds to the region where both effects are equally important. There is a region (shown in white) where the temperature rise exceeds 80 K (assuming an initial temperature of 293 K) and thus boiling would be expected to occur. An estimate of the time required for the fluid to reach boiling temperatures in 2DiDEP constrictions with various buffer concentrations and applied RMS voltages obtained using the 1D transient analytical formula (Equation A5) is shown in Figure 5.13.

5.7 Summary

Until recently, linear electrokinetics has been assumed in most of insulator-based electrokinetic systems for particle and flow manipulations. In this chapter, we provide experimental evidence of recirculating particle trajectories near insulating constrictions influenced by nonlinear electrokinetic effects (ICEO, electrothermal, DEP, and combinations thereof). We demonstrate the effects of insulating constriction geometries, an important iDEP system design factor, on the interplay of these nonlinear electrokinetic phenomena. The relative importance of these effects is highlighted through analytical expressions for ICEO and electrothermal velocities obtained via scaling arguments, which show good agreement with COMSOL simulations. We illustrate the distinguishable characteristic flow patterns of ICEO and electrothermal flow in both 2D and 3D cases that can be used to quickly identify the dominant mechanisms in experimental electrokinetic systems. In addition, by investigating the relationships of multiple variables (constriction ratio, ionic strength, particle size, and applied electric field) on the interplay of these nonlinear electrokinetic effects, we demonstrate that electrothermal and ICEO effects can dominate over DEP under a wide range of circumstances often used in iDEP systems. In a more general sense, this study and the scaling analysis can be useful for designing electrokinetic systems to mitigate (e.g. cell concentration and separation) or exploit (e.g. mixing) these effects on particles and biological cells.
Chapter 6

Conclusions and Future Work

In this thesis, a microfluidic device employing 3DiDEP was utilized to study two interesting bacterial envelope phenotypes – extracellular electron transfer (EET) and lipopolysaccharide (LPS) biosynthesis. A three-step electrical phenotyping workflow, including 3DiDEP-based cell trapping to determine the critical electric field, PIV-based measurement of cell linear electrokinetic mobility, and cell shape measurement, was established to estimate the cell surface polarizability of bacteria. Many existing iDEP-based approaches distinguish bacteria based on size or cell viability, which can also be achieved with high sensitivity and throughput using other techniques, e.g. deterministic lateral displacement or dean flow. The method presented in this thesis allows sensitive detection of small phenotypic variations on bacterial cell envelopes.

First, this approach was applied to achieve rapid and sensitive phenotyping of EET in G. sulfurreducens, S. oneidensis, as well as E. coli strains. Cell surface polarizability was introduced for the first time as a novel parameter to provide a quantitative characterization of several EET-related phenotypes, including the presence/type/amount of crucial outer-membrane cytochromes, the presence and metabolic state of a specific EET conduit, as well as the form of electron acceptors in the growth medium. Additionally, the positive correlation observed between G. sulfurreducens polarizability and its power production in a microbial fuel cell (MFC) suggests exciting potentials for evolutionary selection of electrochemically active microorganisms for optimal current production using cell surface polarizability as a proxy.

Second, the 3DiDEP approach was applied to characterize E. coli mutants with different LPS compositions. E. coli strains with a ‘deep rough’ LPS phenotype require increased trapping voltage for 3DiDEP cell immobilization compared to the strain with full-length LPS, whereas strain expressing the additional O-antigen region results in decreased trapping voltage. The inverse
correlation between trapping voltage and bacterial LPS composition suggests opportunities of rapid and noninvasive characterization of LPS using microfluidic DEP.

Last, the nonlinear electrokinetic phenomena, including the interplay of electrothermal and induced-charge electroosmosis (ICEO), was analyzed from a both experimental and theoretical viewpoint for iDEP systems. Electrothermal and ICEO flows are potential confounding factors that interfere the precision and efficiency of DEP-based particle manipulations. Analytical approximations of electrothermal and ICEO velocity scales were provided as a quick guide for the design of iDEP systems.

Results of this study can be useful as guidance for further DEP-based screening of various bacterial envelope phenotypes for a diverse array of cells and microorganisms. Besides the two bacterial envelope phenotypes (electron transport and LPS biosynthesis) studied in this thesis, other surface features such as the presence of ion channels may also correlate with cell surface polarizability. Additionally, our results suggest opportunities to select bacteria with optimized cell envelope phenotype by integrating the cell polarizability-based screening into the process of directed evolution. [177] Directed evolution requires high-throughput screening methods for identifying phenotypic variants with desired properties. Widely-used screening methods include spatially separated variants as colonies on solid media (or transferred into multiwell liquid culture plates) in combined with chromatography, mass spectrometry or nuclear magnetic resonance (NMR) for product formation analysis, but this technique imposes a practical throughput limit of fewer than $\sim 10^4$ library members per screening round. [177] Alternatively, fluorescence-activated cell sorting (FACS) provides high-throughput isolation of cells containing desired gene variants, but it requires luminescent proteins within cells or cell surface-displayed epitopes. The 3DiDEP-based screening platform can be a potential label-free screening method for iterative selection of microorganism for desired phenotype (e.g. electrochemical activity).

To improve the efficacy and broaden the application of the 3DiDEP-based screening platform, it is necessary to scale up the system for a higher throughput. As discussed in Chapter 2.1, the $VE^2$ dependence causes the DEP force to be short-range, i.e. the DEP force is significant only in the
close vicinity of the electric field non-uniformity, and thus limiting high-throughput operations in DEP-based systems. One way of surmounting this challenge is to extend the single 3DiDEP constriction into a microarray consisting of multiple staggered rows of constrictions (Figure 6.1), which allows iterative exposure of DEP on the cells passing through the microarray, and thereby potentially enhances cell trapping efficiency. Figure 6.1 shows as a proof of concept that *E. coli* cells can be depleted from the fluid stream using a microarray of 3DiDEP constrictions.

Moreover, the current study on the DEP-based phenotyping of bacterial electrochemical activity and LPS composition uses laboratory strains. However, many environmental bacterial and archaea strains are capable of extracellular electron transfer, and are challenging to analyze using conventional phenotyping methods. For future work, it is proposed to evaluate EET microbes from environmental samples and LPS strains with clinical relevance to further assess the efficacy of the 3DiDEP electrical phenotyping platform.

Figure 6.1 **High-throughput bacterial cell separation based on cell surface polarizability using a 3DiDEP microarray.** a) A schematic of the cell separation system. A 1.5 cm by 6.8 mm (length by width) microarray of 3DiDEP constrictions was built in a prototype PDMS chip to enable a pressure-driven flow (5 μL/min). b) A schematic diagram showing that bacteria with stronger surface polarizability will be trapped near the constrictions by DEP, while cells with lower surface polarizability pass through. c) Top view of the estimated cell trajectories in the vicinity of a single constriction by a 3D numerical model. d) and e) Micrographs showing *E. coli* cell motion before (d) and during the application of an AC potential difference (1 kHz, 500 Vrms) across the microchannel. The microarray contours are shown in red.
Appendix

A-1 Nonlinear electrokinetic velocity measurement in iDEP systems

Before each experiment, a dynamic surface modification was conducted to stabilize surface charge on the inner channel walls following the procedure presented in Section 2.2.1 I. Suspensions of 1 μm yellow-green FluoSpheres (Invitrogen, Eugene, OR) in potassium chloride were introduced into the 2DiDEP microchannel using a syringe from the stainless-steel needle on one side of the microchannel. Then, the two syringe needles on either side of the microchannel were connected by tygon tubing (McMaster Carr, Princeton, NJ) to balance the pressure at the two outlets (Figure 5.1). Sinusoidal voltage signals with 1 kHz frequency and various electric field intensities (200 ~ 900 V/cm RMS in the microchannel opening regions) were applied to the stainless-steel needles by a 33220a Agilent Waveform Generator (Agilent Technologies, CA, USA) coupled with a TREK 623B High Voltage Amplifier (TREK, NY, USA). The velocity field in the 2DiDEP constriction was obtained by processing the fluorescent images of microbead motion using a digital particle image velocimetry (PIV) MATLAB package [174] (Figure 5.5c). Additionally, particle trajectories and their velocities along pathlines (Figure 5.5a and 5.5b) were determined using ImageJ 1.51a with a 2D particle tracking algorithm [173]. Particle tracking experiments with 200 nm microbeads did not show significant difference from the results with 1 μm beads (Figure 5.4), suggesting that 1 μm beads behave as passive tracers in these 2D experiments and DEP on the beads is negligible compared to ICEO/electrothermal effects. In the experiments with 3DiDEP microchannels, suspensions of 200 nm red and 1 μm yellow-green FluoSpheres (Invitrogen, Eugene, OR), and 6 μm red fluoresbrite microspheres (Polysciences, Warrington, PA) in 1 mM potassium sulfate media were used (Figure 5.10).
A-2 Analytical approximation of transient temperature at microchannel constriction center and in regions far from constriction

Within the first few minutes after the application of electric fields, the Fourier number (Fo) for the microchannel PDMS domain (5 mm thick) is small, indicating unsteady heat conduction in the PDMS domain. At the constriction center, the fluid temperature reaches the maximum, and thermal conduction along the channel axial direction in the fluid domain is zero due to the symmetry. Therefore, at the constriction center, Joule heating of the electrolyte and conduction in the transvers direction can be viewed as heating of an electric cable, with a heat generation density of

\[ q'' = \langle \sigma_f E \cdot \bar{E} \rangle, \]

buried in a PDMS sheath with infinite thickness. Same approximation can be made at regions far from the constriction, where heat conduction in channel axial direction in the fluid domain is also small (Figure 5.7c). Because the cross-section of the fluid domain has an area much smaller than that of the PDMS domain, we approximated the heat-generating region to be circular with an equivalent radius \( r_0 \), and solve the problem of heat conduction in composite cylindrical regions (Figure A-1) using the Laplace transformation method proposed by Carslaw and Jaeger [172]. For the case far from the constriction, \( r_{0,\text{far}} = W_0 / \pi \), because heat transfer occurs mainly at the top and bottom microchannel walls. For the case of constriction center, \( r_{0,\text{center}} = \beta r_{0,\text{far}} \). Initially, temperature is zero (relative to 293 K) in both of the fluid and PDMS domains. For time \( t > 0 \), heat is generated at the constant rate \( q'' \) per unit time per unit volume in the region \( 0 \leq r < r_0 \). The heat conduction equation can be expressed as

\[
\frac{d^2 T_1}{dr^2} + \frac{1}{r} \frac{dT_1}{dr} - \frac{1}{\alpha_f} \frac{\partial T_1}{\partial t} = -\frac{q''}{k_f}, \quad 0 \leq r < r_0 \quad (A1)
\]

\[
\frac{d^2 T_2}{dr^2} + \frac{1}{r} \frac{dT_2}{dr} - \frac{1}{\alpha_w} \frac{\partial T_2}{\partial t} = 0, \quad r > r_0 \quad (A2)
\]
where \( k_f, \alpha_f \) and \( k_w, \alpha_w \) are the thermal conductivity and thermal diffusivity of the fluid and PDMS domains, respectively. Using the Laplace transform, 
\[
L\{T(r,t)\} = \overline{T} = \int_0^\infty e^{-st}T(r,t)dt
\]
Equation A1 and A2 can be rewritten as
\[
\begin{align*}
\frac{d^2 \overline{T}_1}{dr^2} + \frac{1}{r} \frac{d \overline{T}_1}{dr} - p_1^2 \overline{T}_1 &= -\frac{q'''}{k_f s}, \quad 0 \leq r < r_0 \\
\frac{d^2 \overline{T}_2}{dr^2} + \frac{1}{r} \frac{d \overline{T}_2}{dr} - p_2^2 \overline{T}_2 &= 0, \quad r > r_0
\end{align*}
\]
where \( p_1 = \sqrt{s/\alpha_f} \) and \( p_2 = \sqrt{s/\alpha_w} \). Assuming no contact resistance at the boundary \( r = r_0 \), the boundary conditions there are \( \overline{T}_1 = \overline{T}_2 \), and \( k_f \frac{d \overline{T}_1}{dr} = k_w \frac{d \overline{T}_2}{dr} \). Additionally, Equation A3 and A4 have to be solved subject to the condition that \( \overline{T}_1 \) is finite at \( r = 0 \), and that \( \overline{T}_2 \) is bounded as \( r \to \infty \). The solutions are transformed using the Inversion Theorem as
\[
\begin{align*}
\frac{(T_1 - T_0)k_w}{q'''r_0^2} &= 4 \int_0^{\tilde{r}} \left[ J_0(\tilde{\lambda} r) J_1(\tilde{\lambda}) \frac{\lambda^2}{\lambda^2(\varphi^2 + \psi^2)} \right] d\lambda, \quad \tilde{r} < 1 \\
\frac{(T_2 - T_0)k_w}{q'''r_0^2} &= 2 \int_0^{\tilde{r}} \left[ J_0(\tilde{\lambda} r) J_1(\tilde{\lambda}) \left[ J_0(A\lambda \tilde{r}) \varphi - Y_0(A\lambda \tilde{r}) \psi \right] \frac{\lambda^2}{\lambda^2(\varphi^2 + \psi^2)} \right] d\lambda, \quad \tilde{r} < 1
\end{align*}
\]
Here, \( \text{Fo}_1 \) is the Fourier number of the fluid domain, i.e. \( \text{Fo}_1 = \alpha_f t/r_0^2 \). Parameters \( \varphi, \psi, \) and \( A \) are defined as
\[
\begin{align*}
\varphi &= KJ_1(\lambda)Y_0(A\lambda) - AJ_0(\lambda)Y_1(A\lambda) \\
\psi &= KJ_1(\lambda)J_0(A\lambda) - AJ_0(\lambda)J_1(A\lambda) \\
A &= \sqrt{\alpha_f/\alpha_w}, \quad K = k_f/k_w
\end{align*}
\]
where \( J_n(\cdot) \) and \( Y_n(\cdot) \) are the Bessel functions of the first kind and second kind, respectively, with order \( n \). The dimensionless parameter, \( \tilde{r} = r/r_0 \). The time course of the temperature rise at constriction center and regions far from the constriction (Figure 5.7b) were estimated using Equation A5 at \( \tilde{r} = 0 \) with \( r_0 = r_{0,\text{center}} \), \( q''' = \sigma_f E_0^2 / \beta^2 \) and \( r_0 = r_{0,\text{far}} \), \( q''' = \sigma_f E_0^2 \), respectively.

Figure A-1 **Transient temperature at microchannel constriction center and opening regions was estimated using a 1D heating and conduction model in composite cylindrical regions.** This is a schematic of the microdevice cross-sectional view, where the circular fluid domain \( (0 \leq r < r_0) \) generates heat with a density of \( q''' \) per time per volume, surrounded by an infinite PDMS domain. Here \( k_f, \alpha_f, k_w, \alpha_w \) are the thermal conductivity and thermal diffusivity of the fluid and PDMS domains, respectively.


