ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

GRAM-POSITIVE BACTERIA SENSING IN A MICROFLUIDIC CHIP BY AC ELECTROPHORESIS

M.Sc. THESIS

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Nanoscience and Nanoengineering Department

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Merve YALÇIN, a M.Sc. student of ITU Graduate School of Science Engineering and Technology with student ID 513151020, successfully defended the thesis entitled "GRAM-POSITIVE BACTERIA SENSING IN A MICROFLUIDIC CHIP BY AC ELECTROPHORESIS" which prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

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To my family,

FOREWORD

I would like to thank my supervisor Prof. Dr. Hüseyin KIZIL for this kind support and his guide. That was a good opportunity work under his supervisor. The experience I achieved will lead my future works. I also thank to Prof. Dr. Nevin Gül Karagüler for this kind support and because of allowing for using her lab. Besides, I thank to my lab friends especially İpek İrem Aykın and Kadriye Ölmez. Working with them and conducting experience together was both enjoyful and instructive. Finally, but not last, sincerely thanks to my dear family for their help and support me in every decision I made.

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ABBREVIATIONS

AC	**:** Alternating Current
CM	**:** Clausius-Mossotti
cfu	**:** Colony Forming Unit
DEP	**:** Dielectrophoresis
EO	**:** Electroosmosis
EDL	**:** Electrical Double Layer
EOF	**:** Electroosmotic Flow
IA	**:** Impedance
IDAM	**:** Interdigitated Array Microelectrodes
LOC	**:** Lab-on-a-chip
NS	**:** Navier-Stokes
n-DEP	**:** Negative Dielectrophoresis
OSHA	**:** Occupational Safety and Health Administration
PVD	**:** Physical Vapor Deposition
PDMS	**:** Polydimethylsiloxane
PCR	**:** Polymerase Chain Reaction
p-DEP	**:** Positive Dielectrophoresis
KOH	**:** Potassium Hydroxide
TS	**:** Tryptic Soy
TSB	**:** Tryptic Soy Broth
UV	**:** Ultraviolet

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AC ELEKTROFOREZ İLE MİKROAKIŞKAN SİSTEMLERDE GRAM-POZİTİF BAKTERİ ALGILAMASI

ÖZET

Mikroakışkan sistemler, küçük ölçekli boyutlara sahip ince kanallarda akışkanların yönlendirildiği sistemdir. Biyolojiden kimyaya, optikten bilgi teknolojisine birçok farklı alanda çeşitli uygulamaların yapılmasına olanak sağlayan seçkin bir alandır. Deneylerin süresini kısaltarak daha hızlı sonuçlar elde etme, yüksek maliyetli reaktiflerin daha az kullanılmasıyla toplam maliyeti azaltma, moleküllerin ayrılmasında ve saptanmasında yüksek hassaslıkla birlikte yüksek çözünürlük sağlama ve çok küçük hacim ve boyutlarda az enerji tüketimi ile yüksek oranda akışkan kontrolünün sağlanması gibi önemli avantajlar sağlar. Kanseri ve patojenleri saptamak amacıyla diyagnostik deneylerde kullanılabilir ve kapiler elektroforez, DNA analizi, akış sitometrisi, imünolojik testler, PZR amplifikasyonu ve hücrelerin ayrılması gibi birçok farklı alanda kullanılabilir. Bu sistem çip-üstü-laboratuvar olarak da bilinir. Çip-üstü- laboratuvar araçları ve isteğe uyarlanmış elektronik birim mikroakışkan çip üzerinde dielektroforez (DEP) ve empedans (IA) yöntemlerini birleştirir.

DEP, elektrokinetik kuvvetlerin alt gruplarından biridir ve düzgün olmayan elekriksel alanda yüklü ya da nötr parçacıkların elektriksel hareketi olarak tanımlanır. Bu yöntemi kullanarak çeşitli biyosensör uygulamaları için bakterinin konsantre edilmesi ve bakteri kayıplarını azaltmakla birlikte akış hızını düşürerek hızlıca mikroorganizmaların tespit edilmesine olanak sağlar. Bu yetenekler sadece bakteriler ile sınrlı olmamakla birlikte mayalar, virüsler, DNA, kanser hücreleri, kırmızı ve beyaz kan hücreleri, karbon nanotüp gibi parçacıklar ve 105 Da dan fazla kütleye sahip proteinler için de geçerlidir. Empedans ise elektrokimyasal metodlardan biridir ve parçacıkların elektriksel özelliklerine dayanır. Çip-üstü-laboratuvar araçları empedans ölçümlerini reseptörleri, enzimleri, kan hücrelerini, DNA'yı, antikorları, virüsleri, makrofajları, fibroblastları ve endotelyal hücreleri konsantre etmek ve saptamak amacıyla kullanır. Empedans tekniği kullanılarak Gram-pozitif ya da Gram-negatif birçok farklı bakteri türü tespit edilip niceliği belirlenebilir. Örneğin, *Salmonella, E. coli, Listeria innocua, Staphylococcus aureus, Enterococcus faeccalis* ve *Listeria monocytogenes.* Saptama süresi saniyelerden 1 güne kadar değişkenlik gösterebilir.

Bakteri hücrelerini agar plaka üzerinde kültüre etme ve görüntüleme gibi geleneksel yöntemler en az bir gün olmakla birlikte haftalarca sürebilir. Bu yöntemlerle yapılan deneylerde, *E. coli* gibi hızlı büyüyen bir bakteri için analiz süresi bir-iki gün arasında değişkenlik gösterirken *Mycobacteria* gibi yavaş büyüyen türlerde analiz süresi birkaç hafta sürebilir. Bununla birlikte bazı bakteri türleri mikroskop altında gözlemlenebilir, fakat kültüre edilemez. Hayatı tehdit eden enfeksiyonlarda ve acil durumlarda bu analiz süresi çok uzun olacaktır ve bu kabul edilemez. Analiz süresini azaltmak amacıyla, bakteriyi konsantre etme, saptama ve tanımlama işlemleri kültüre etme ve büyüme adımları olmaksızın gerçekleştirilmelidir. Bu amaçla, bu çalışmada

DEP ve IA yöntemleri kullanılmıştır. Bu yötemlerin birleştirilmesiyle hücrelerin tutulması, saptanması ve parçalanması ile birlikte DNA, RNA, mayalar, virüsler, kanserli hücreler ve bakterilerin saptanması kısa sürede gerçekleştirilmiş olacak.

Bakteriler prokaryotik hücrelerdir. Bu hücreler tek halkalı DNA molekülüne sahip olmakla birlikte çekirdek ve membran-bağlı organelleri eksiktir. Bakteriler hücre zarının yapısına bağlı olarak iki gruba ayrılırlar: Gram-pozitif bakteriler ve Gram-negatif bakteriler. Gram-negatif bateriler, dış zara sahip olmakla birlikte ince bir peptidoglikan tabakasına sahiptirler. Gram-pozitif bakteriler ise dış zara sahip olmamakla birlikte kalın bir peptidoglikan yapısına sahiptir. Gram-pozitif bakteri iki farklı empedans bölgesine sahiptir; yüksek impedans bölgesi hücre zarında bulunurken alçak impedans bölgesi hücre duvarında bulunur. AC elektrik alan mikroakışkan sistemler içinde bakteri hücrelerine uygulandığında bu impedans bölgeleri arasında gidip gelir. Bununla birlikte, Gram-pozitif bakteriler hücre duvarlarında anyonik özellik gösteren teikoik asitlere sahiptir. Bu anyonic özellik AC elektrik alanını etkiler.

Bu çalışmada, Gram-pozitif bakterilerden *Enterococcus faecalis* kullanıldı. Her bakteri farklı ve kendine has elektriksel özelliğe sahip olduğu için DEP ve IA metodlarıyla deney süresini kısaltarak çok düşük konsantrasyonlarda bile bu bakterileri konsantre etmek ve saptamak mümkün olacaktır.

GRAM-POSITIVE BACTERIA SENSING IN A MICROFLUIDIC CHIP BY AC ELECTROPHORESIS

SUMMARY

Microfluidics is a study of systems which manipulate fluids in tiny channels with dimensions at microscale. It is a distinct field which provides making applications in different fields from biology and chemistry to optics and information technology. Microfluidic systems provide several advantages in these fields especially curtailing time of experiments and by this way, giving faster results, reducing whole costs because of lesser use of overpriced reagents and providing high resolution as well as sensitivity in both detection and experiments of separation of molecules, and accomplishing greater fluid control with low energy consumption under small volumes and small size. It can be utilized in a wide range of applications: In order to detect cancer and pathogens, it can be used in diagnostic experiments and utilized in different procedures like capillary electrophoresis, DNA analysis, flow cytometry, immune assays, PCR amplification and separation of cells. These systems also known as Lab-on-a-chip system. Lab-on-a-chip devices and an electronic unit, which is customized, combines DEP and IA on the microfluidic chips.

Dielectrophoresis is one of the subgroups of electrokinetic force, and is described as the movement of electrically charged or neutral particles in a non-uniform electric field. By using DEP method, various capabilities are performed like concentrating, arraying, rotating and moving particles as well as biological materials. DEP is a bright field in order to concentrate bacteria by using several biosensor applications, since by using DEP, it is possible to detect microorganisms rapidly by reducing flow rates as well as reducing bacteria losses. These abilities are not just specific for bacteria but for yeasts, viruses, cancer cells, red and white blood cells, DNA, proteins with a mass of higher than 105 Da, and particles like carbon nanotubes, as well.

Impedance is one of the electrochemical methods, which is based on the electrical features of particles. Lab-on-a-chip (LOC) tools utilize the impedance measurements in order to concentrate and detect receptors, enzymes, blood cells, DNA, antibodies, viruses, macrophages, fibroblasts, endothelial cells and so on. Besides, single cell impedance analysis are utilized effectively for a number of techniques like cell counting, discrimination, behavior analysis and growth of bacteria. By using the impedance technique, it is possible to detect and quantify several species of bacteria which are either gram-positive or gram-negative like *Salmonella, E. coli, Listeria innocua, Staphylococcus aureus, Enterococcus faeccalis, Listeria monocytogenes* and so on. Detection time vary from seconds to 1 day.Traditional methods like culturing and visualizing of bacterial cells on an array of agar plates takes time of one day to a few weeks. In order to identify bacteria by these methods, analysis time is one to two days for *E. coli* growing fastly, while analysis time is a few weeks for *Mycobacteria* species growing slowly. Besides, some species of bacteria can be viewed under the microscope; however, they are non-culturable. In the circumstances like emergency case and life-threatening infections, analysis time will be so lengthy, and this is not reasonable. So as to diminish analysis time, concentrating, detecting and identifying bacteria must be performed without culturing and growth steps. Instead of these methods, DEP and IA were utilized in this study. As a combination,

utilizing the DEP and IA is very influential in order to detect DNA, RNA, yeasts, viruses, cancerous cells and bacteria as well as to trap, detect and lyse of the cells.

Bacteria are prokaryotes which have single circular DNA as a genetic material but not nuclei and membrane-bound organelles. One of the classification in the bacteria is based on structure of cell envelope which is peptidoglycan thickness. In this classification, two types of bacteria are present: Gram-positive bacteria and Gram-negative bacteria. While Gram-negative bacteria possess thin peptidoglycan layer as well as outer cell membrane, Gram-positive bacteria possess thick peptidoglycan layer, but not outer cell membrane. There are two impedance regions found in the Gram-positive bacteria: high impedance region in cell memrane and low impedance region in the cell wall. When the AC electric field is performed on the bacterial cells within the microfluidic devices, it alternates between these impedance regions. Besides, teichoic acids found in the cell wall of Gram-positive bacteria possess the anionic characteristic property because of the presence of phosphoric acid residues. This anionic property is affected by the AC electric fiels.

In this study, *Enterococcus faecalis* was used that belongs to the type of Gram-positive bacteria. Since each bacteria type possess different and specific electrical properties, it is possible to concentrate and detect a specific bacteria type by the methods of DEP and IA effectively even at very low concentrations in a very short period time.

1. INTRODUCTION

1.1 Microfluidics

Microfluidics is a study of systems which manipulate fluids in tiny channels with dimensions at microscale. Microfluidics is a distinct field which provides making applications in different fields from biology and chemistry to optics and information technology [1].

There are several advantages of microfluidic systems in different fields: diminishes amounts of sample and reagent used in experiments, curtails time of experiments and by this way, gives faster results, reduces whole costs because of lesser use of overpriced reagents, provides very precise flow control because of laminar or smooth flow of fluids in micro channels, provides high resolution as well as sensitivity in both detection and experiments of separation of molecules, ensures better control of experimental parameters along with sample concentration at microscale [1, 2].

In addition, microfluidic channels accomplish greater fluid control with low energy consumption under small volumes (μL, Nl, Pl, Fl) and small size (mm, μm) [2].

Microfluidics is often utilized and defined as “lab on a chip” and “organ on a chip” [2]. However, it can be utilized in a wide range of applications: In order to detect cancer and pathogens, it can be used in diagnostic experiments; utilized in different procedures like capillary electrophoresis, DNA analysis, flow cytometry, immune assays, PCR amplification, separation of cells and so on; antibiotic drug resistant bacteria, investigation of chemical reaction kinetics and nanoparticle transport in blood are main focus studies in the applications of microfluidics. Besides, microfluidics used in pharmaceutical applications with the aim of drug discovery and in cosmetic applications as emulsions and formulations [1].

In fluid mechanics, laminar and turbulent flows are two basic types of flow. Turbulent flow has the properties of violent and chaotic. Compared to turbulent flow, laminar flow has peaceful properties and in the microfluidic system with laminar flow, particles sustain their rota without mixing each other. Whether the flow is laminar or turbulent is determined by Reynolds Number of flowing liquid [3].

Reynold number is defined as the ratio of intertial forces to viscous forces and is the dimensionless number which determines the physical nature of flow. If intertial forces are higher than viscous forces, physical characteristics of flow is turbulent flow (Figure 1.1) [4].

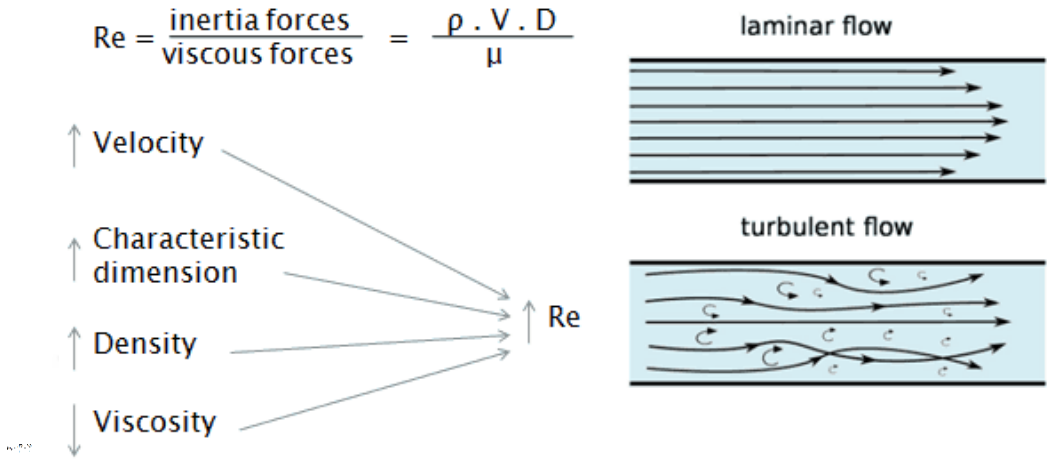


Figure 1.1 : Formulation of Reynold number, and laminar flow versus turbulent flow [4].

In the formula of Reynold number, ρ is fluid density, V is the velocity of flow, D is characteristic linear dimension (hydraulic diameter), and μ is dynamic viscosity [4].

1.2 Electrokinetic Force

Electrokinetic is attributed to study of movement and behavior of charged particles or fluids in suspension based on the applied electrical field [5, 6]. With the advancement in the microfabricated electrode structures, it is possible to force the behavior of particles. High electrical fields generated by this way cause heat loss, and as a result of that, thermal gradients are generated which bring about fluid motion by means of buoyancy and electrothermal forces [5]. The most common subgroups of electrokinetic force are electrical double layer, electrophoresis, dielectrophoresis, electroosmotic force and electrothermal force [6, 7].

1.2.1 Electrical double layer

By the time the solid surfaces interact with ionic aqueous solution, these surfaces get surface charges. Charged surface interact with the opposite ions and repel co-ions in the fluid as a consequence of electrostatic interaction. This leads to forming a thin layer of opposite ions near the charged surface. This layer is named as electrical

double layer (EDL) including two layers which are stern and diffuse layers (Figure 1.2) [8].

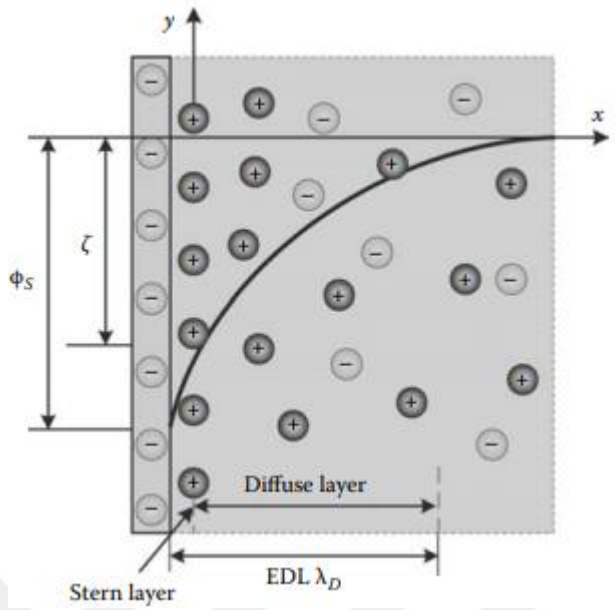


Figure 1.2: Electrical double layer [8].

Because of much strong electrostatic force, ions in the stern layer have not ability to move while ions in the diffuse layer have the ability to move. Therefore, net charge is originated in the electric potential in the diffuse layer. Thickness of EDL depends on the Debye length (λ_D) which is related with the bulk concentration of ionic solution (Figure 1.2) [8].

1.2.2 Electrophoresis

Electrophoresis is defined as moving of charged colloidal particles under applied electrical field. Because of electric surface charge of these colloidal particles, they have an advantage of freely suspended in an electrolyte solution. However, they do not take an advantage of conducting electricity. An electrostatic (Coulombic) force is applied on the charged colloidal particles and on the counter ions in double layer. Since the whole system formed from particles and ions in the diffuse double layer is neutral, net electrostatic body force is zero. Because of that, not the electric force but the "slipping" motion of double layer moves these colloidal particles. Hence, it is thought as "force free" motion. Consequently, particles move with a velocity which is proportional to electric field. Through the electro-osmotic flow, particles move around it (Figure 1.3) [6].

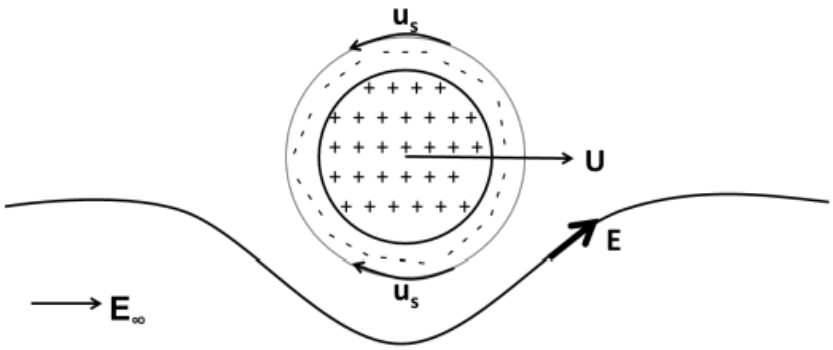


Figure 1.3: Motion of charged colloidal particles because of applied electric field [6].

1.2.3 Dielectrophoresis

In a uniform electric field, electrically charged and neutral particles act differently because of different ion/charge distribution on the particles. In this field, electrically charged particle move away from the electrode with same charges and get closer to electrode with opposite charges because of Coulomb force. In contrast, neutral particle gets redistributed charges, and constitute a dipole moment. Coulombic forces on opposite sides of dipole moment formed on this particle are equal to each other. Hence, net force is zero [9]. The process realized in the uniform electric field describes the electrophoresis phenomena (Figure 1.4_a) [10]. However, in a non-uniform electric field, Coulombic forces acting on the neutral particle forms a net force which is not zero. This force is named as Dielectrophoretic force [9]. Therefore, Dielectrophoresis is described as the movement of electrically charged or neutral particles in a non-uniform electric field (Figure 1.4_b) [9, 10].

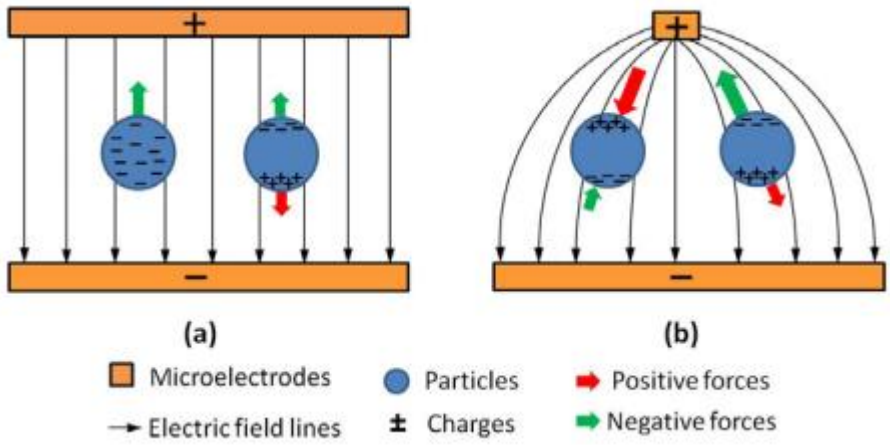


Figure 1.4: a) Electrophoresis vs. b) Dielectrophoresis.

Dielectrophoretic force exerting on a spherical, homogeneous particle in non-uniform electric field is shown as

$$\vec{F}_{DEP} = 2\pi r^3 \varepsilon_m Re[f_{CM}]\nabla(\vec{E}^2_{rms}) \quad (1.1)$$

where r represents the radius of spherical particle, $\nabla(E^2_{rms})$ represents the gradient of squared electric field, ε_m shows the electrical permittivity of the suspended media, and Re represents the real part and $[f_{CM}]$ represents the Clausius-Mossotti (CM) factor. The subscript rms of the squared electric field is made sense of root mean square and is feasible for electric fields produced by AC power sources [10]. Besides, Clausius-Mossotti factor is represented by the equation of

$$[f_{CM}] = \frac{\varepsilon_p^* - \varepsilon_m^*}{\varepsilon_p^* + 2\varepsilon_m^*} \quad (1.2)$$

where ε^* represents the complex electrical permittivity, and subscript p and m represent the particle and media, respectively [10]. E^* is also formulated by

$$\varepsilon^* = \varepsilon - \frac{j\sigma}{2\pi f} \quad (1.3)$$

where σ, f and j represent the electrical conductivity, frequency of electric field and root mean square of negative one ($j = \sqrt{-1}$), respectively [10] .

With regards to dipole moment, p, dielectrophoretic force is formulated by the equation of

$$\mathbf{F}_{DEP} = \mathbf{p}\nabla\mathbf{E} = 2\pi\varepsilon_m r^3 \mathbf{Re}[K(\omega)]\nabla\mathbf{E}^2 \quad (1.4)$$

This equation shows that DEP force is affected by several parameters like particle size, spatial non-uniformity of electric field, and permittivity and conductivity of both of particle and suspending media. Furthermore, DEP force is also influenced by CM factor (f_{CM} or K(ω)) which stands for dielectric features of DEP environment by contributing the particle and media. This CM factor also shows that the frequency of electric field affects the DEP force. That is, when the particle is much more than the media in terms of polarizability (Re[f_{CM}] or Re[K(ω)]>0), dielectrophoretic force on the particle push the particle towards the region of high electric field. This type motion is named as positive DEP (p-DEP) (Figure 1.5). On the contrary, when the media is much more than the particle in terms of polarizability), dielectrophoretic force on the particle pushes the particle away from the region of high electric field. This type motion is named as negative photoresist (n-DEP) (Figure 1.5) [9, 10].

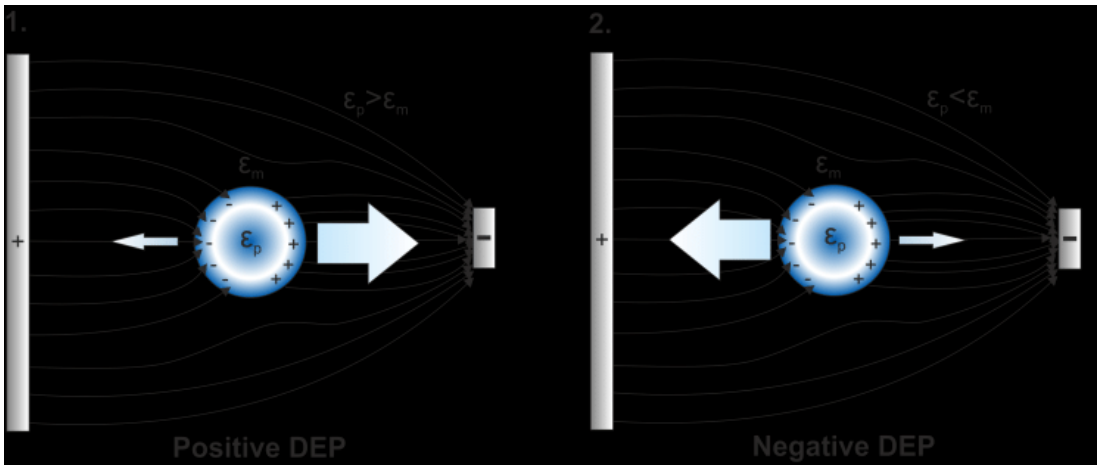


Figure 1.5: p-DEP (left) and n-DEP forces (right) in a non-uniform electric field. Big arrows show the direction of DEP forces [11].

By using DEP method, various capabilities are performed like concentrating, arraying, rotating and moving particles as well as biological materials. DEP is a bright field in order to concentrate bacteria by using several biosensor applications, since by using DEP, it is possible to detect microorganisms rapidly by reducing flow rates as well as reducing bacteria losses. These abilities are not just specific for bacteria but for yeasts, viruses, cancer cells, red and white blood cells, DNA, proteins with a mass of higher than 105 Da, and particles like carbon nanotubes, as well [12].

Despite there are various advantages of DEP over other methods, it possesses some disadvantages. Microorganisms is affected by their surroundings and media. Properties of medium like pH, temperature, conductivity and electrolyte valency influence the particle. Besides, reagents found in media can be absorbed by the surface of the particle. This makes compulsory to control the external factors so as to obtain consistent DEP results. Another important factor is to modify surface charge of particle before checking the alterations in the behavior of DEP. Because of complicated electronic control devices and discrepancy with heterogeneous sample matrices, integration of DEP with micro-devices induce the another challenge [12].

1.2.4 Electro-osmotic force

Electro-osmosis (EO) is a basic electrokinetic effect which is the movement of liquid as a consequence of mutual effect between electrical double layer (EDL) and applied electric field [13]. This phenomena also named as electroosmotic flow (EOF) [8]. The charges on the surface interact with opposite ions in the solution and repel the

same ions within the microfluidic channel. This lead to EDL near the surface. By applying electric field, the charges in the EDL bring about a net Coulomb force, and as a result of that, these charges migrate throughout the microchannel. Then, dragging of the bulk fluid within the channel happens. As a consequence, fluid flows with a consistent velocity within the microchannel [13]. The electrokinetic force having an impact on the liquid is formulated by the equation of

$$\mathbf{F} = \mathbf{E}\sum_{i=1}^{n} Fz_i c_i = -\varepsilon_0 \varepsilon_f \nabla^2 \phi \mathbf{E} \tag{1.5}$$

where E in the equation is external exerted electric field. Besides, by the Navier-Stokes (NS) equation, the movement of fluid is also written as

$$\rho\left(\frac{\partial \mathbf{u}}{\partial t} + \mathbf{u} \bullet \nabla \mathbf{u}\right) = -\nabla p + \mu \nabla^2 \mathbf{u} - \varepsilon_0 \varepsilon_f \nabla^2 \phi \mathbf{E} \tag{1.6}$$

where ρ, u, *p* and μ are density of fluid, velocity of fluid, pressure and fluid dynamic viscosity, respectively (Figure 1.6) [8].

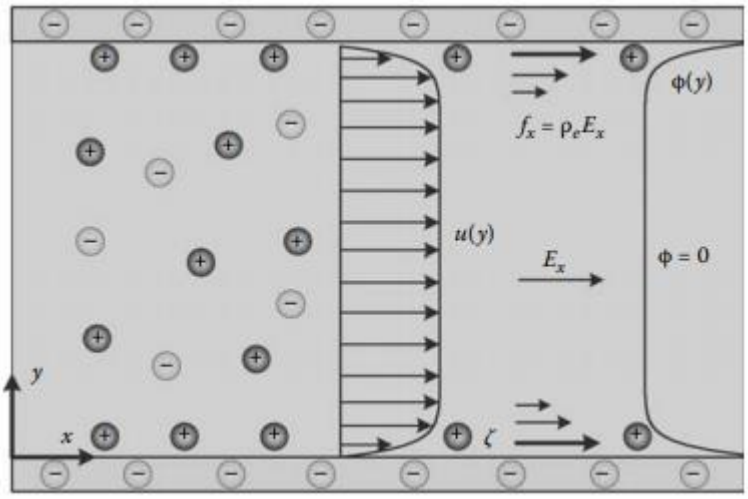


Figure 1.6: Schematic representation of EOF modified by Navier-Stokes equation [8].

There are several applications of EOF in order to move fluids in the microfluidic tools like drug delivery, microelectronics cooling, high performance liquid chromatographic separations, microinjection systems and so on [8].

1.2.5 Electrothermal force

Electrothermal flows are generated when the small particles are managed by the high electric fields. As a consequence, large power density is produced within the fluid around the electrode. Equation of power per unit volume is written as

$$W = \sigma E^2 \ (\mathrm{W\ m^{-3}}) \tag{1.7}$$

where σ and E are conductivity and electric field, respectively. Large temperature enhancement happens as a result of large power produced in so small volume. Energy balance equation is utilized to determine this temperature enhancement for a given electrode, which is

$$\rho_m c_p \bar{\boldsymbol{v}} \cdot \nabla T + \rho_m c_p \frac{\partial T}{\partial t} = k\nabla^2 T + \sigma E^2 \tag{1.8}$$

where v, T, ρ_m, c_p, k and σ are velocity, temperature, mass density, specific heat capacity, thermal conductivity and electrical conductivity, respectively [5].

Not only electric field but also power density is quite non-uniform. Therefore, local variations in conductivity, permittivity, density and viscosity of medium is caused by local gradients in the temperature of liquid like water. These variations bring about to forming net forces on the fluid. Buoyancy force and electrothermal force are two types of these forces. Buoyancy force happens if the variations in fluid density are generated through the variations in the temperature, and this lead to natural convection. In contrast, electrothermal force happens if the local changes in the medium are generated through the variations in conductivity and/or permittivity associated with the temperature. Local changes in the conductivity generates both free volume charge and Coulomb force whereas local changes in the permittivity generates dielectric force [5, 14].

1.3 Impedance

One of the electrochemical methods is impedance which is based on the electrical features of particles. Impedance analysis arises from bio-affinity incidents concluding alterations in resistance and capacitance over either the surface of electrode or the surface of a substrate [12]. In impedance analysis, impedance of each particle as well as total impedance is formulated by the equations of

$$\frac{1}{|Z|} = \frac{1}{|Z_1|} + \frac{1}{|Z_2|} \tag{1.9}$$

$$|Z_1| = \sqrt{R_{sol}^2 + \frac{1}{(\pi f C_{dt})^2}} \tag{1.10}$$

$$|Z_2| = \frac{1}{2\pi f C_{de}} \tag{1.11}$$

where f, R_{sol}, C_{dt}, and C_{de}, stands for frequency, solution resistance, double layer capacitance and dielectric capacitance of solution, respectively while Z_1, Z_2 and Z represents the impedance of R_{sol} and C_{dt}, impedance of C_{de} and total impedance which arise from parallel Z_1 and Z_2 [12].

In impedance analysis, an excitation signal depending on frequency is introduced into the system, and as a result, measurement of response is performed. As an excitation signal, alternating current (AC) voltage signal, generally sinusoidal (U(ω)) is applied to the system, and as a consequence, measurement of electric current response is performed [15]. Complex impedance of the system is formulated by the equation of

$$Z(\omega) = \frac{U(\omega)}{I(\omega)} = Z_{RE} + jZ_{IM} \tag{1.12}$$

where $j=\sqrt{-1}$ and ω represents frequency. Z_{RE} and Z_{IM} are the real and imaginary parts of the complex impedance, respectively. Z_{RE} is named as resistance while Z_{IM} is reactance. Formulas of the magnitude (|Z|) and phase angle (θ) of this complex impedance are written as [15]

$$|Z| = \sqrt{(Z_{RE})^2 + (Z_{IM})^2} \tag{1.13}$$

$$\theta = \arctan\left(\frac{Z_{IM}}{Z_{RE}}\right) \tag{1.14}$$

Impedance depending on frequency are much useful and efficient technique in order to characterize the cells and their compartments not only in micro-/nano fluidic systems but also macro fluidic systems. Hence, there are various biological areas so as to concentrate biological materials [12]. Detection with impedance has some advantages like simple to design, high sensitivity, non-invasive, label-free electrochemical method and obtaining quantitative results [12, 16]. However, high

impedance of probe electrodes, EDL and stray capacitance restrict the right measurement of biological features of cells within the microfluidic system [12].

In the impedance technique, in order to determine impedance change (ΔR) which gives the measurement, passing of a particle by a gap between two electrodes is necessary. Microfluidic channel includes this gap as inbuilt. In order to obtain high ΔR/R ratio, electrodes must be so close to one another and current lines must be concentrated on the cell [17]. Measurements are obtained as signal by detecting the impedance difference ($Z_{AC} - Z_{BC}$), when the cell passes through the electrodes in the fluid shown in the Figure 1.7 [17, 18].

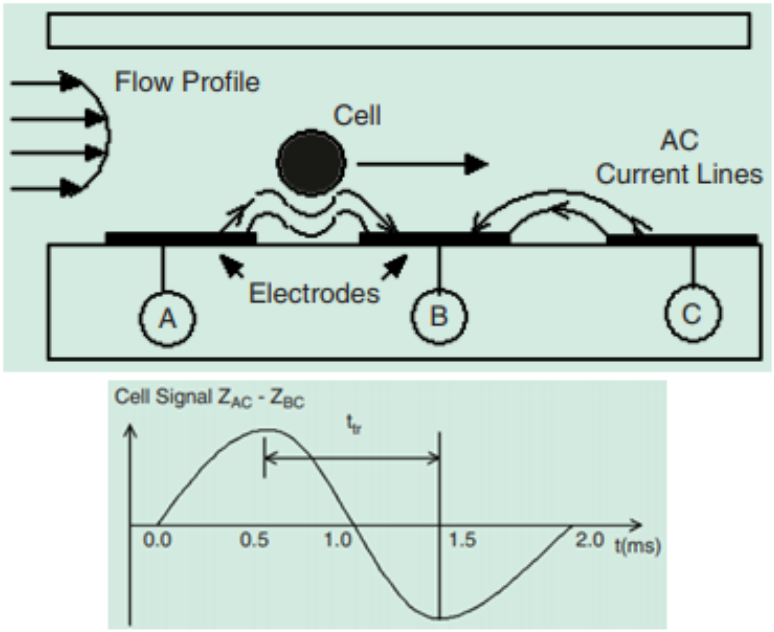


Figure 1.7: Schematic represantation of microchannel (above) and impedance difference signal (below) [17].

Besides, electrical model of impedance change is indicated at Figure 1.8. Through this model, it is possible to determine the impedance change analytically either analytically or simulated by utilizing 3D finite elements [17].

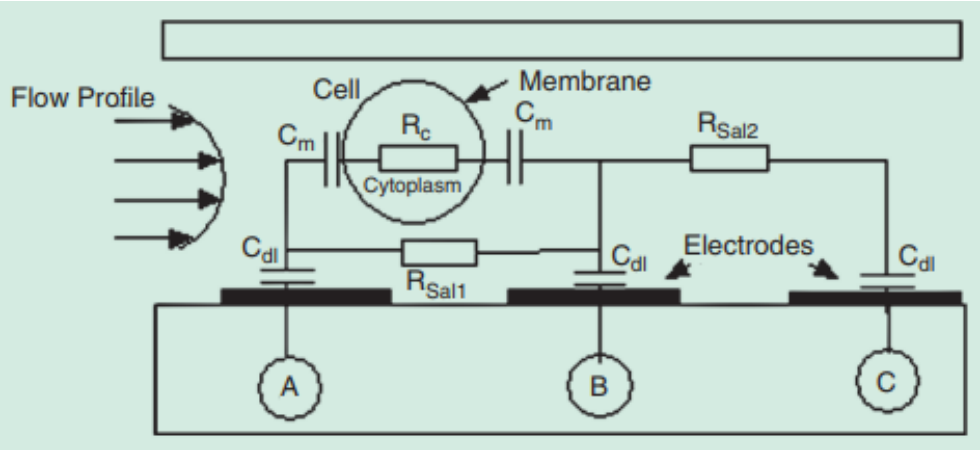


Figure 1.8: Electrical model of impedance change [17].

Lab-on-a-chip (LOC) tools utilize the impedance measurements in order to concentrate and detect receptors, enzymes, blood cells, DNA, antibodies, viruses, macrophages, fibroblasts, endothelial cells and so on. Besides, single cell impedance analysis are utilized effectively for a number of techniques like cell counting, discrimination, behavior analysis and growth of bacteria. By using the impedance technique, it is possible to detect and quantify several species of bacteria which are either gram-positive or gram-negative like *Salmonella*, *E. coli*, *Listeria innocua*, *Staphylococcus aureus*, *Enterococcus faeccalis*, *Listeria monocytogenes* and so on. Detection time vary from seconds to 1 day [12].

1.4 Dielectrophoresis and Impedance

Traditional methods like culturing and visualizing of bacterial cells on an array of agar plates takes time of one day to a few weeks. In order to identify bacteria by these methods, analysis time is one to two days for *E. coli* growing fastly, while analysis time is a few weeks for *Mycobacteria* species growing slowly. Besides, some species of bacteria can be viewed under the microscope; however, they are non-culturable. In the circumstances like emergency case and life-threatening infections, analysis time will be so lengthy, and this is not reasonable. So as to diminish analysis time, concentrating, detecting and identifying bacteria must be performed without culturing and growth steps [16, 19, 20].

Instead of these methods, DEP and IA are utilized in this study. Lab-on-a-chip devices and an electronic unit, which is customized, combines DEP and IA on the

microfluidic chips (Figure 1.7_a). Firstly, sample is concentrated in customized electronic unit by

DEP force (Figure 1.7_b), and then response for this concentrated sample is obtained by using IA monitors (Figure 1.7_c) [12].

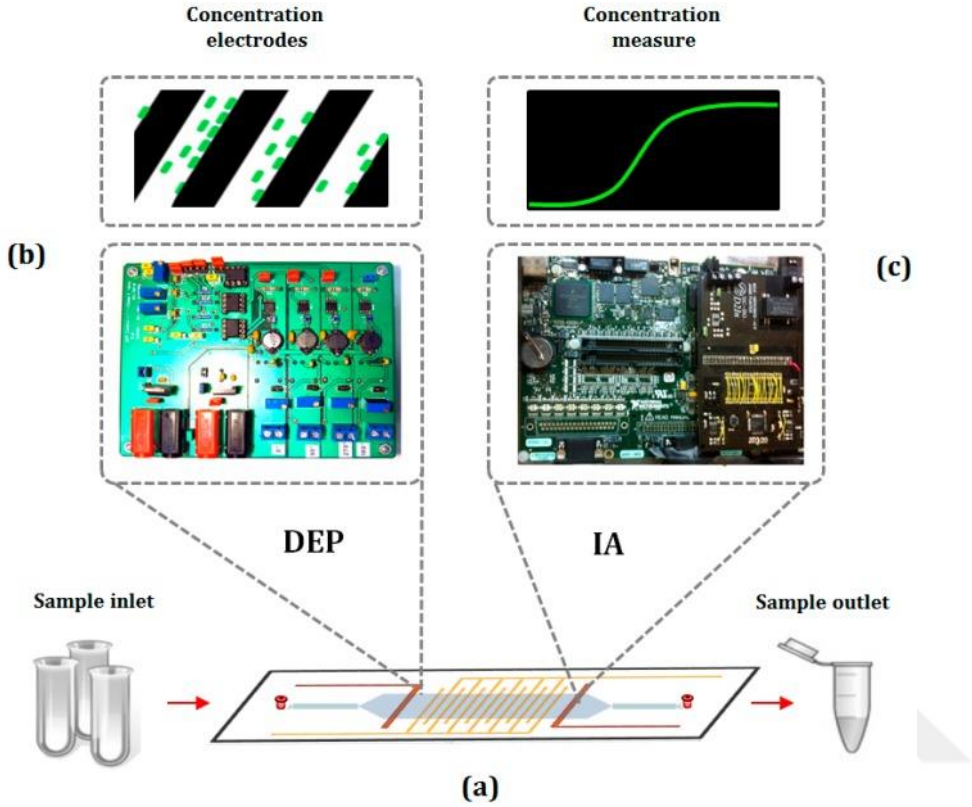


Figure 1.9: Schematic representation of combined DEP and IA approach. a) Microfluidic chip; b) Concentration of bacteria by DEP; c) Measurement of concentration by impedance analysis [12].

Low frequency capacitance is changed by DEP because of concentration of particle over the electrodes. When the DEP force hold the cells at interdigidated array microelectrodes (IDAM), an equivalent volume of the medium is replaced by its permittivity. As a result, changing in the complex permittivity of the medium alter the impedance between electrodes, and the outcome is given as a graph. At high frequency ranges, impedance analysis is useful for single-cell cytometry [12].

As a combination, utilizing the DEP and IA is very influential in order to detect DNA, RNA, yeasts, viruses, cancerous cells and bacteria as well as to trap, detect and lyse of the cells. To concentrate and detect bacteria, some methods are shown in the Table 1.1 [12].

Table 1.1: Literature Review (NA: no data available)

Author	Principle	Buffer	Conductivity	Bio-Affinity Element	Applied Frequency	Flow Rate Conditions	Bacteria	Sample Rate	Concentration	Signal Variation
Suehiro et al (2003)	DEP + IA	Manitol Solution	0.2 mS/m	Polyclonal Antibodies	1 MHz	9×10^2 µL/min	*E.coli* strain K12	NA	10^7 cells/mL	NA
Suehiro et al (2005)	EPA-DEP + IA	DI water	0.2 mS/m	No Element	100 kHz	5×10^2 µL/min	*E.coli* strain K12	NA	10^4 to 10^2 CFU/mL	NA
Sabounchi et al (2008)	iDEP + IA	DI water	1-2 µS/cm	Fluorescent Beads (2 µm)	100 Hz	40 µL/min	*B. subtilis* spores	10 µL/min	10^6 spores/mL	NA
Hamada et al (2013)	nDEP-pDEP + IA	Manitol Solution	0.1 mS/m	No Element	1 kHz (nDEP) and 100 kHz (pDEP)	0.27 m/s	*E.coli* strain K12 (NBRC3301)	NA	NA	NA
Dastider et al (2013)	pDEP + IA	PBS Solution and DI Water	Low	Polyclonal Antibodies	100 Hz-1 MHz	2-4 µL/min	*E.coli* O157:H7	3×10^5 CFU/mL	3×10^2 CFU/mL	NA
Del Moral-Zamora et al (2015)	DEP + IA	Milli-Q Water	0.5×10^{-3} to 2.5×10^{-3} S/m	No Element	500 Hz to 5 kHz	10 µL/min	*E.coli* 5K strains	NA	2×10^7 cells/mL	3.1 %
Couniot et al (2015)	DEP + IA + (AC-EO)	PBS at pH 7.4	1.8 mS/m	No Element	10 kHz-63 MHz	5 µL/min	*S. epidermidis* ATCC 35984	NA	3.5×10^5 CFU/mL and 3.8×10^6 CFU/mL	NA

1.5 Bacterial Cells

Bacteria are prokaryotes which have single circular DNA as a genetic material but not nuclei and membrane-bound organelles [21]. Because of that, their DNA is found in cytoplasm. Besides, because of not having nucleus, all of the mechanisms including transcription process which transcribe segments of DNA into RNA, and translational process which translate the codons into protein sequence, take place simultaneously [21, 22]. Cytoplasm and cell envelope are two significant compartments in the bacteria. In addition, they may consist of moving part in the exterior of cell like flagella and pili (Figure 1.8) [22, 23].

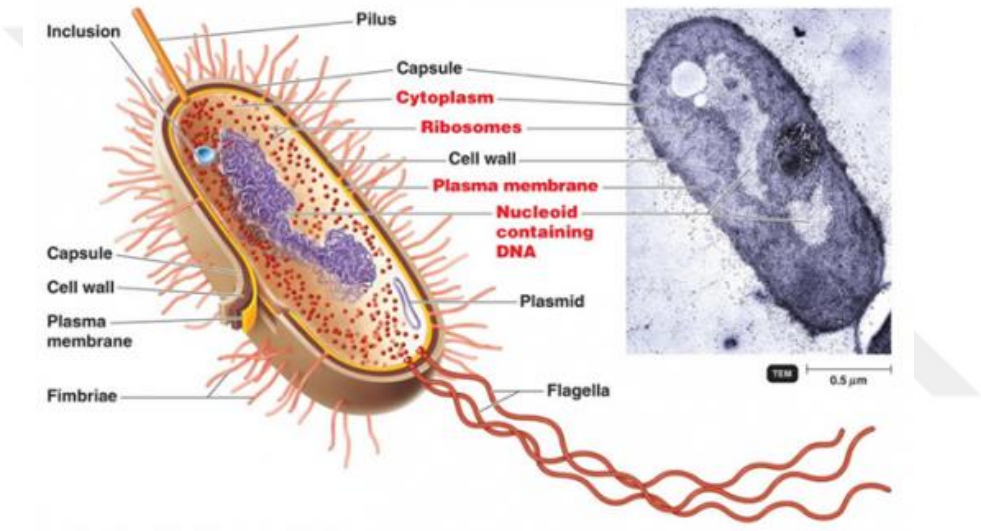


Figure 1.10: Bacterial cell structure [25].

Bacterial cells have different shapes and sizes from each other. Rod, spherical and curve shapes are the shapes generally seen in the bacteria, and named as bacilli, cocci and spirilla, respectively [24]. Based on the size, *Mycoplasmas* is the smallest bacteria which is 0.1-0.25 micrometers in diameter whereas *Thiomargarita namibiensis* is one of the biggest bacteria which is 750 micrometer in diameter and can be observed by naked eye [22, 25].

Another classification in the bacteria is based on structure of cell envelope. All bacteria include the bilayer membrane which encircles cytoplasm. In the bilayer membrane, there are integral membrane proteins in order to transport sugars and aminoacids into the cell for growth. There is peptidoglycan structure above the bilayer membrane in the extracellular environment, and this structure is seen in the

most bacteria except *Mycoplasma*. Because of the presence of the lipid cholesterol, these bacteria have merely lipid bilayer. Peptidoglycan is a polymer which happen naturally, and comes into existence from chains of glycans (sugar) connected to each other by peptide cross-links. These cross-links are prevented by Beta-lactam antibiotics like penicillin and ampicillin [22]. There are two types of bacteria based upon the structural differences in their cell walls. In order to distinguish these bacteria from each other, Hans Christian Gram proposed a test. Based on this test, if the bacteria withhold the crystal violet dye after washing with alcohol and water because of thick peptidoglycan layer, they are named as Gram-positive bacteria [22, 26, 27]. Compared to these bacteria, Gram-negative bacteria do not withhold crystal violet dye after washing with alcohol and acetone because of thin peptidoglycan layer, and can be decolorized in order to accept Safranin as a counter stain. As a result, they are viewed as pink or red color under the microscope [26, 27].

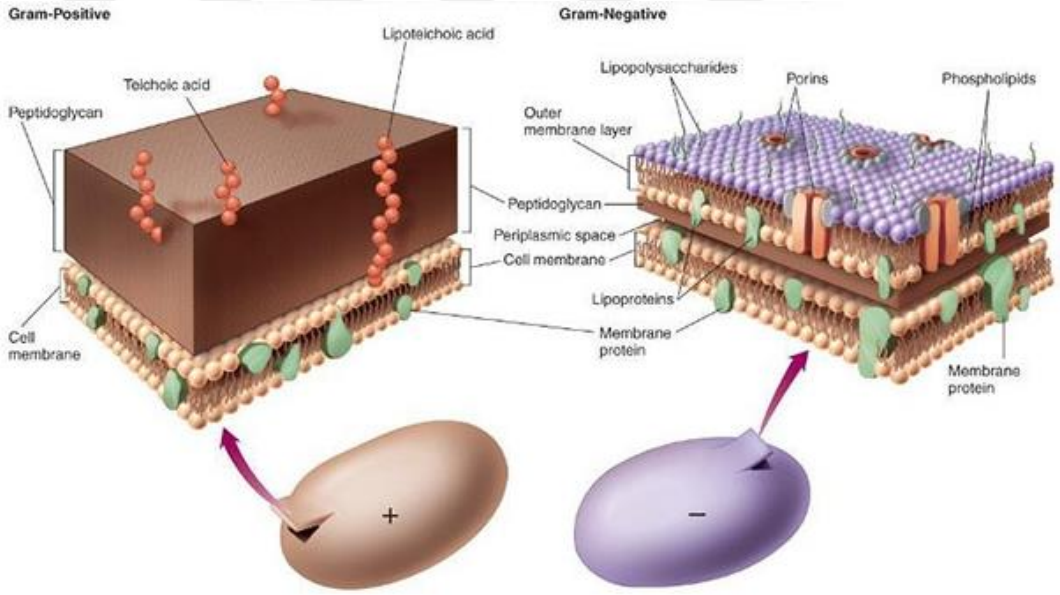


Figure 1.11: Gram-positive vs. Gram-negative bacteria [28].

Gram-positive bacteria possess thick peptidoglycan layer and so that, thick cell wall which play role as molecular barrier in order to hinder the harmful molecules to reach the cytoplasmic membrane and damage to the cell. Compared with that, Gram-negative bacteria possess thin peptidoglycan layer, but possess outer membrane as a second bilayer membrane. This outer membrane protects the cell like in Gram-positive bacteria. In the outer membrane, Gram-negative bacteria include porin proteins which transport the small molecules like sugar, salts and peptides from outside the cell into the periplasmic space. Lipopolysaccharide molecules constitute

the outer membrane, and mammalian immune system recognizes these molecules as a microbial product (endotoxin). In the mammalian bloodstream, lipopolysaccharides can be lethal to living being. In the Gram-positive bacteria, this feature belongs to the lipoteichoic acids found in their cell walls [22].

Not only Gram-negative bacteria but also Gram-positive bacteria can be pathogenic. Pathogenic Gram-positive bacteria are *Streptococcus, Staphylococcus, Corynebacterium, Listeria, Bacillus* and *Clostridium* which bring about diseases in humans, and *Rathybacter, Leifsonia* and *Clavibacter* which give rise to diseases in plants. Besides, most of the Gram-negative bacteria like *Pseudomonas aeruginosa, Neisseria gonorrhoeae and Yersinia pestis* bring about diseases. In addition, due to possess lipopolysaccharides in the outer membrane, Gram-negative bacteria are more resistant to a large number of antibiotics than Gram-positive bacteria. Therefore, non-pathogenic Gram-positive bacteria are utilized in different fields commercially like industrial manufacture of amino acids, degradation of hydrocarbons, manufacture of enzymes, cheese ageing and so on [26].

Teichoic acids found in the cell wall of Gram-positive bacteria have extensive structural diversity. Because of the presence of phosphoric acid residues, teichoic acids possess the anionic characteristic property, and these residues decide their functions in the cell like ion exchange and checking over the activity of autolytic enzymes. Besides, teichoic acids attend to interact of bacteria with other microorganisms, plants, animals, proteins and antibodies [29].

One of the Gram-positive bacteria is *Enterococcus faecalis* (Figure 1.12) which can live in different environments like hot, salty and acidic settings but commensaly live in the gastrointestinal tract of humans. [30, 31]. However, when *Enterococcus* species spread to other parts which is present in the body in the case of poor healthy circumstance and/or weakened immune system, they bring about serious infections like sepsis, endocarditis and meningitis [31]. *Enterococcus* species resist a broad array of antimicrobial agents [30]. This comes from the natural ecological settings of *Enterococcus* species. Like the other commensals living in the gastrointestinal tract of humans, exposion to a number of antibiotics during the medical treatment is highly observed in these species. Therefore, they have significant role in the ecological dynamics because of antibiotic resistance. Besides, as an opportunistic pathogen, *Enterococcus faecalis* bring about a variety of life-threatening infections in the settings like hospital [32].

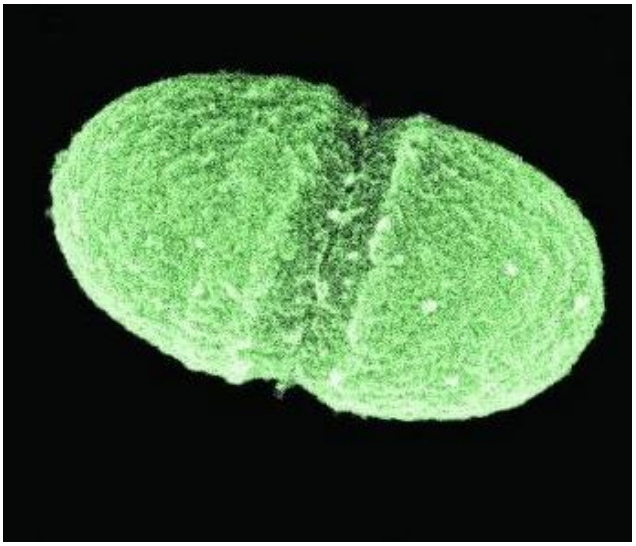


Figure 1.12: *Enterococcus faecalis* [33].

2. EXPERIMENTAL SETUP

2.1 Design

Microfluidic device was created with two parts: focusing region and detection region. To create this device, microchannels and electrodes were drawn by using AUTOCAD 2017 programme. In the channel design, channel height is 25 µm, while the channel width is 300 µm in the focusing region and 100 µm in the detection region.

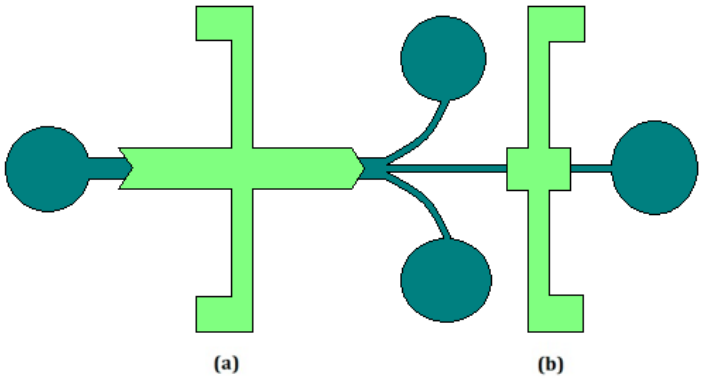


Figure 2.1: Schematic represantation of microfluidic device. a) Focusing region. b) Detection region.

Two types of electrodes were used in the microfluidic devices: focusing region electrode and detection region electrode. Focusing region electrode forms from 100 pairs of electrodes. In each pair, electrode width is 15 µm and gap is 10 µm. There are four different detection region electrodes. Each forms from 25 pair. Width of electrodes and gap between electrodes are 15 µm and 10 µm, respectively.

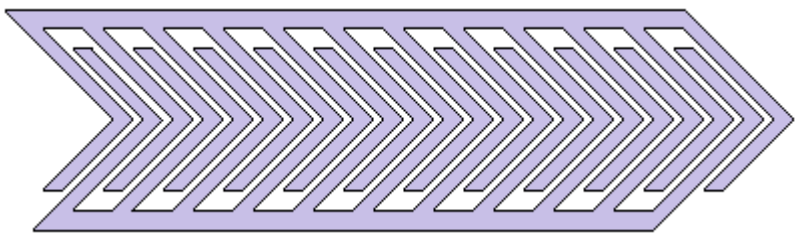


Figure 2.2: Schematic represantation of focusing region electrode.

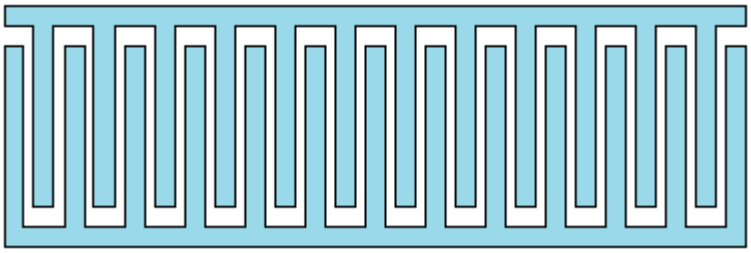


Figure 2.3: Schematic represantation of detection region electrode.

2.2 Fabrication

Firstly, glass slides were obtained in desired sizes by glass cutter. For cleaning of glass slides, they were exposed to potassium hydroxide solution (KOH) for 10 minutes within the ultrasonic bath. After they were washed with distilled water and dryed off with nitrogen gas, they were exposed to acetone solution for 10 minutes within the ultrasonic bath. They were washed with isopropil alcohol and then distilled water, and dryed off with nitrogen gas. After that, glass slides were spin coated by using AZ9260 positive photoresist, and soft bake process was realized on these glass slides at 110°C for 120 seconds. Then, they were exposed to UV light through the lithography machine and AZ400K developer at the proportion of 1:4. Mask design used in the photolithography process were written by utilizing Heidelberg DWL mask writer device. After that, physical vapor deposition (PVD) process was performed in order to deposit titanium on the glass substrates in a vacuum chamber.

Microfluidic channels were fabricated by utilizing SU-8 negative photoresist and polydimethylsiloxane (PDMS). In order to obtain microfluidic channel with 25 μm thickness, this negative photoresist was spin coated over the silicon wafer. PDMS was mixed with curing agent (10:1), and bubble formation was prevented in vacuum chamber. After that, PDMS mixture was poured on the silicon wafer with negative photoresist. PDMS with silicon wafer was put on a hot plate having 90°C for 20 minutes. After the PDMS was separated from Silicon wafer, it was joined with glass substrates with Ti deposited by utilizing plasma cleaner. PDMS-glass substrate was put on a hot plate having 45°C for 10 minutes.

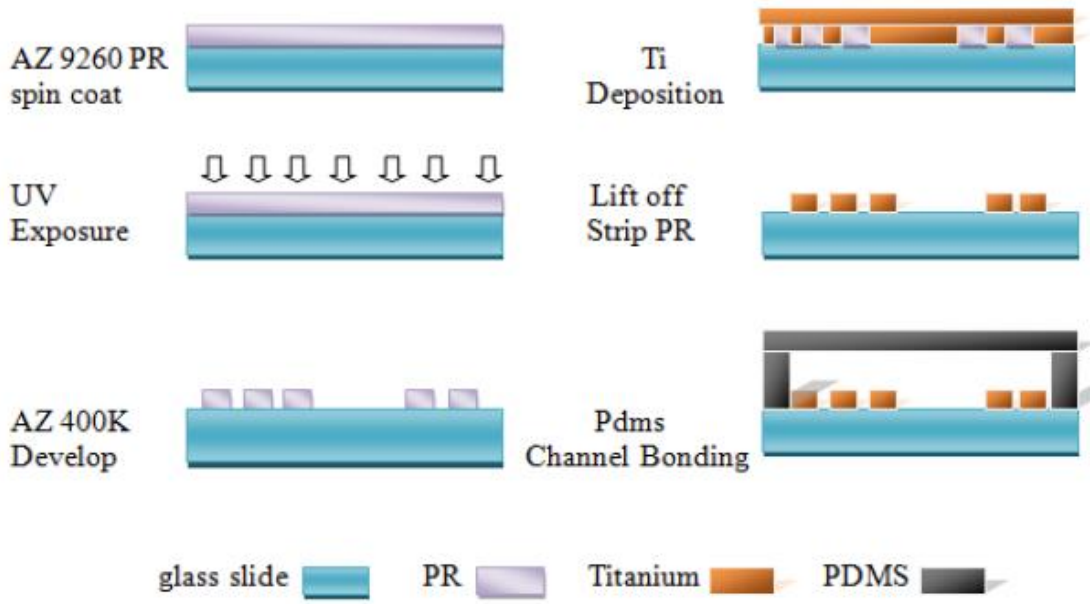


Figure 2.4: Schematic representation of fabrication of microfluidic device.

2.3 Culturing Bacterial Cells

For culturing bacteria, agar plates were prepared. For that, Tryptic Soy (TS) Agar and TS Broth medium were prepared based on the Table 2.1, and TS Agar was poured on the plates. After that, bacterial cells were streaked on TS Agar plates, and incubated at 37°C for 24 hours. Single colony taken from TS Agar plates were inoculated in TS Broth medium. They were incubated at 37°C at 180 rpm for 24 hours. Bacterial cells were prepared with desired concentrations.

Table 2.1: Materials and their amounts needed for preparing TS Agar and TS Broth.

TS Agar / 50 mL	TS Broth / 50 mL
1.5 g Tryptic Soy Broth 0.75 g Agar	1.5 g Tryptic Soy Broth

3. RESULTS

Frequency dependent impedance analysis was realized for *Enterecoccus faecalis* at frequency of between 1 kHz to 500 kHz and voltage of 0.4 V. Different concentrations of this bacteria which are 10^3 cfu/ml, 10^4 cfu/ml, 10^5 cfu/ml and 10^6 cfu/ml were acquired based on the experiment of culturing bacterial cells. Impedance analysis results done with these bacterial concentrations were obtained like shown in the Figure 3.1. Based on these results, impedance decreases when the frequency increases.

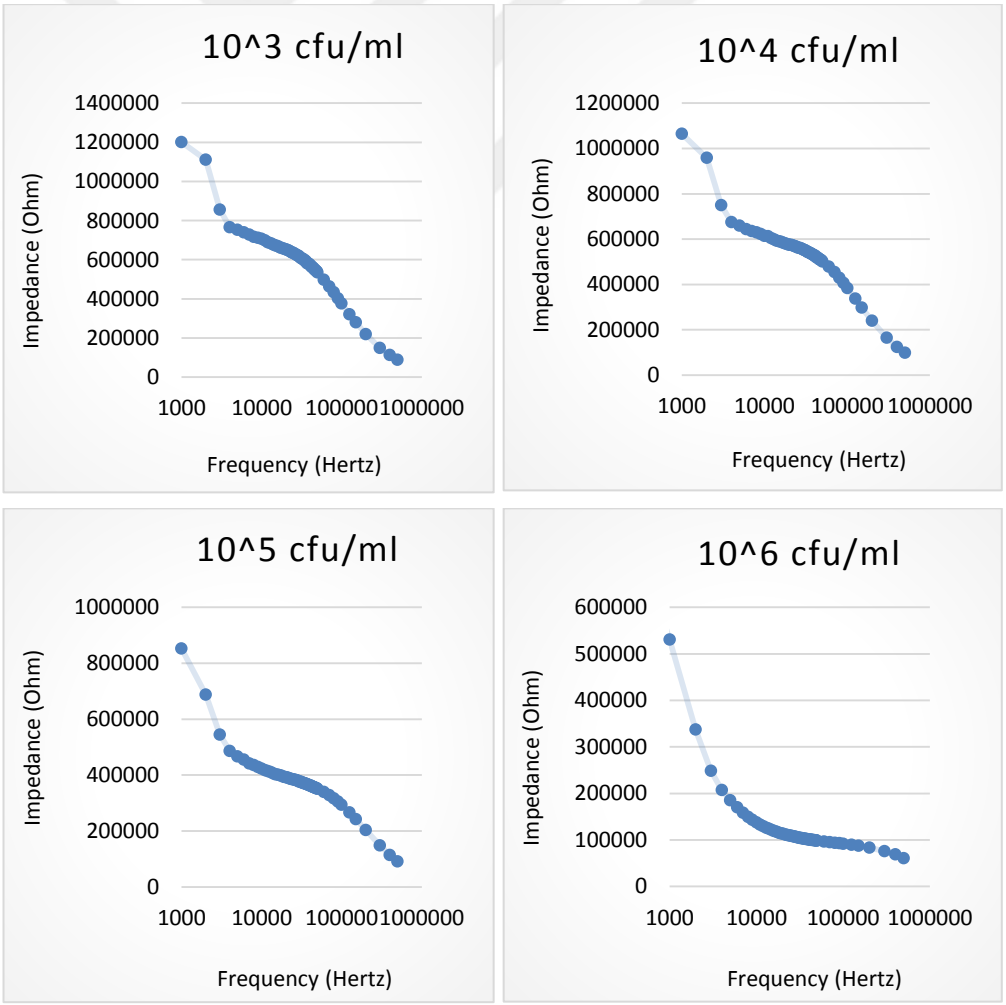


Figure 3.1: Frequency dependent impedance analysis.

Comparisons of frequency dependent impedance values for four different concentrations was shown in the Figure 3.2. This graph shows that impedance decreases when the frequency increases. Besides, this graph shows that impedance also decreases when the concentration increases.

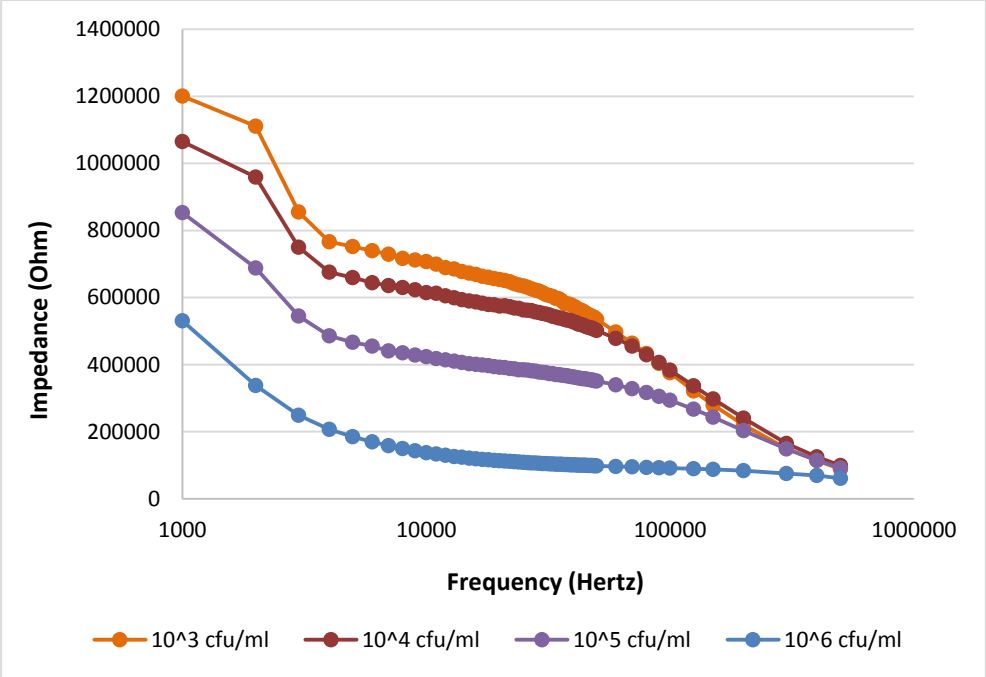


Figure 3.2: Comparisons of frequency dependent impedance responses.

Time dependent impedance analysis was realized for *Enterecoccus faecalis* at the frequency of 10 kHz and voltage of 0.4 V. Different concentrations of this bacteria which are 10^3 cfu/ml, 10^4 cfu/ml, 10^5 cfu/ml, 10^6 cfu/ml, 10^7 cfu/ml and 10^8 cfu/ml were acquired based on the experiment of culturing bacterial cells. Impedance analysis results done with these bacterial concentrations were obtained like shown in the Figure 3.3. Based on these results, impedance decreases when the concentration increases. This is also observed in the Figure 3.4.

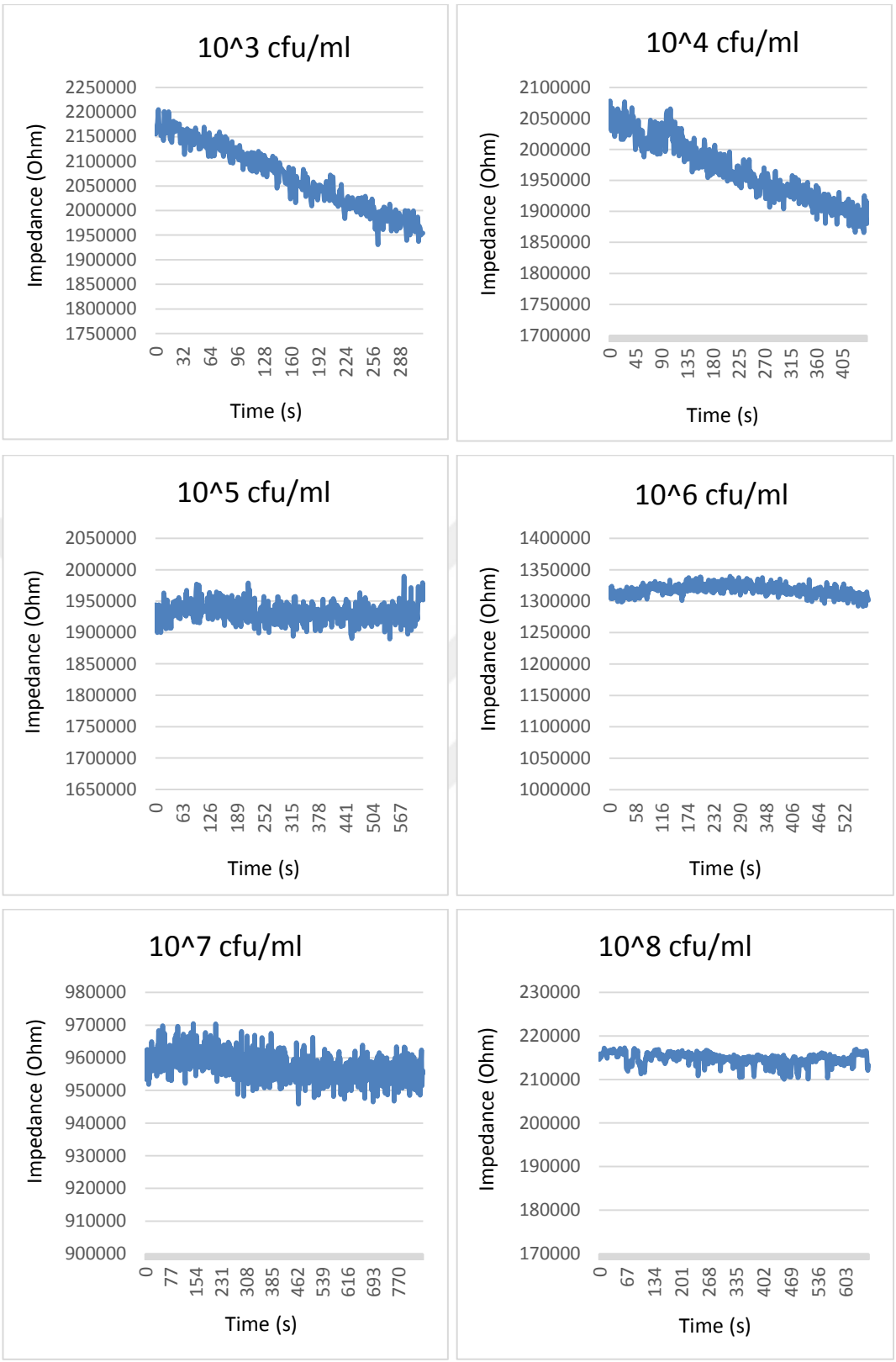


Figure 3.3: Time dependent impedance analysis.

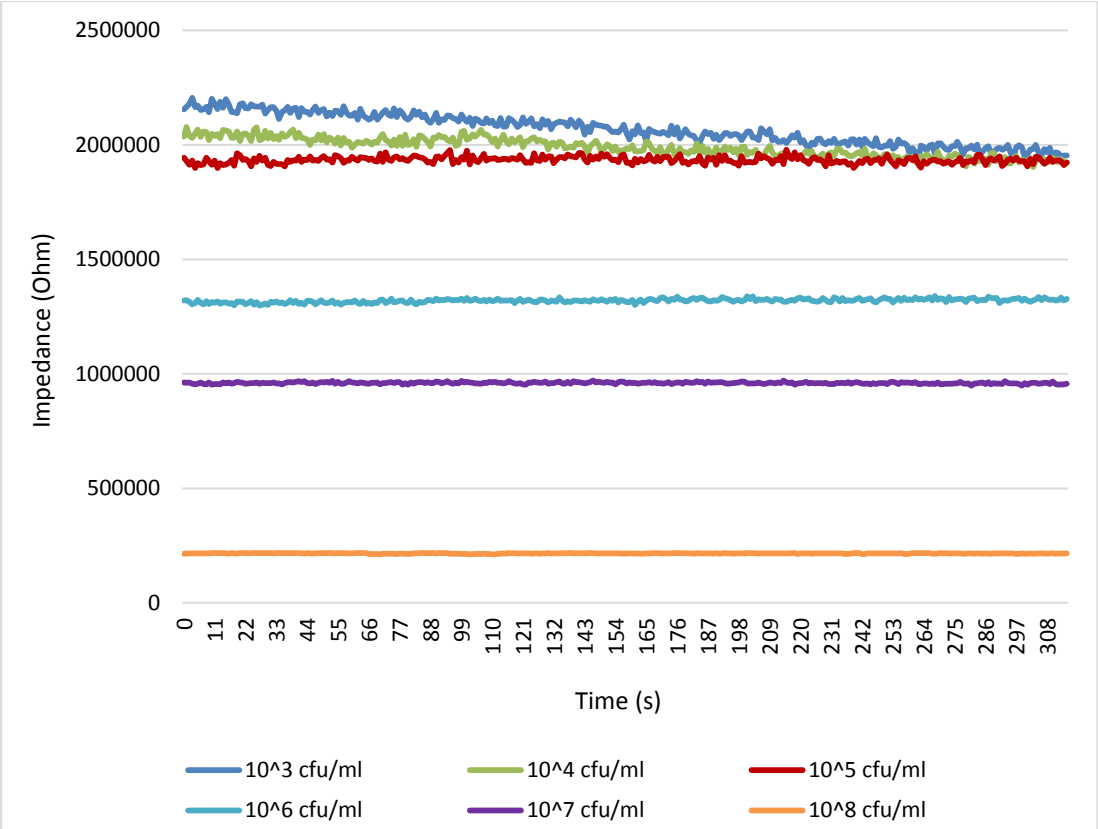
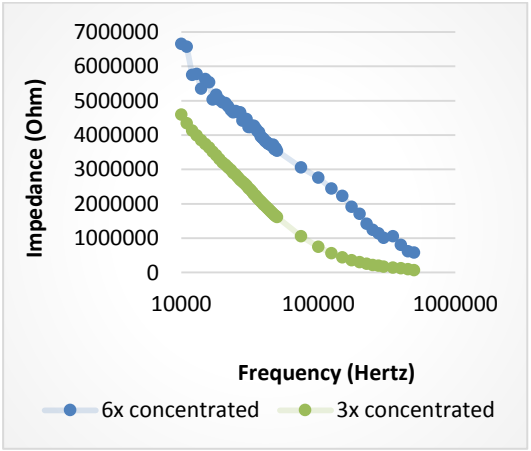


Figure 3.4: Comparisons of time dependent impedance responses.

Polystyrene particles were conducted in distilled water by using DEP and not using DEP, and analysis was realized with two different concentrations which are 3X (3 drops in 10 mL water) and 6X (6 drops in 10 mL water). Depending upon the concentration, polystyrene particles with 6X concentration is two times higher than polystyrene particles with 3X concentration. Based on the dielectrophoretic force and number of particles, impedance decreases like in Figure 3.5 and Figure 3.6. In these experiments, p-DEP was realized at 5 MHz and 5 V.



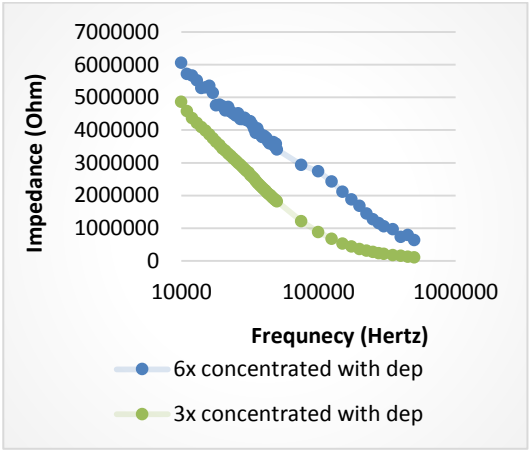


Figure 3.5: Impedance chart without DEP. **Figure 3.6:** Impedance chart with DEP.

Compared to bacteria, in the polystyrene particles, impedance increases with increasing concentration of these particles like shown in the Figure 3.5, Figure 3.6 and Figure 3.7 because of more insulative effect of polystyrene particles than distilled water. In this experiment, the aim is to improve the sensitivity of the biosensor by utilizing DEP. In Figure 3.7, this can be seen but not clearly because of electrodes and their low conductivities.

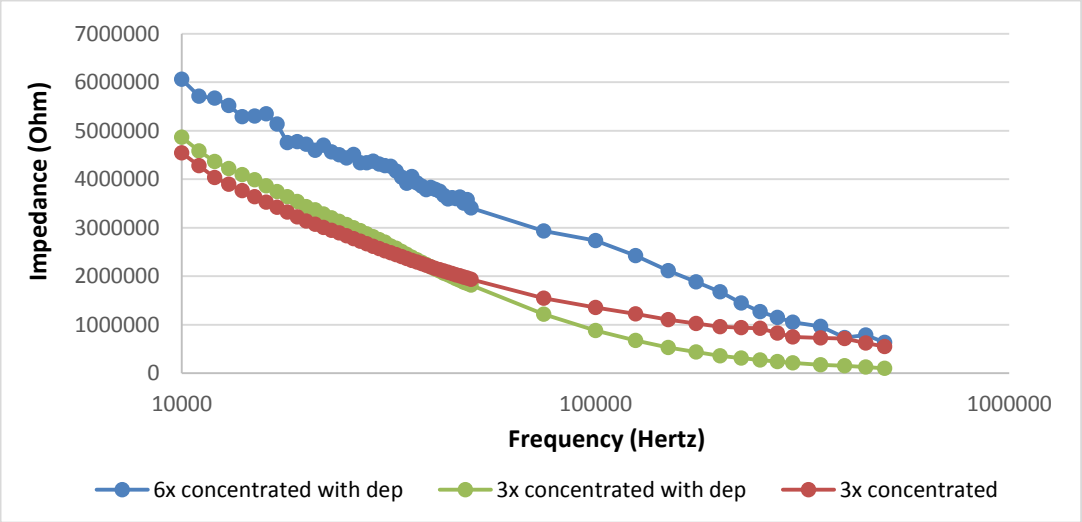


Figure 3.7: Impedance chart of polystyrene particles with 3X and 6X concentrations based on DEP effect and not DEP effect.

4. CONCLUSION

All over the world, diseases originated from bacteria are frequently observed public health problem as well as being costly. For detection of bacteria, traditional methods like culturing and visualizing of bacterial cells on an array of agar plates takes time of one day to a few weeks. In the circumstances like emergency case and life-threatening infections, analysis time will be so lengthy, and this is not reasonable. So as to diminish analysis time, concentrating, detecting and identifying bacteria must be performed without culturing and growth steps. Another important parameter is to detect the bacteria at so low concentrations. For these purposes, instead of these methods, DEP and IA can be utilized as a combination by taking the advantage of different electrical properties of every bacteria. By this way, in the time to come, it may be possible to identify bacteria not utilizing any antibody.

In order to detect the Gram-positive bacteria, *Enteroccus faecalis*, impadance based biosensor was designed and tested. After time and frequency dependent impedance analysis, impedance results were compared to each other. It was found that as low as 10^3 cfu/ml can be detected by this impedance based biosensor. Results revealed that impedance value changes with the bacterial concentration, frequency and the amount of DEP force, which indicates the biosensor is sentitive to these variables and different bacteria types can selectively be detected by this method.

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MESLEKİ DENEYİM VE ÖDÜLLER:

Bildiri

1. Juglonun Salatalıkta (*Cucumis sativus)* Dopa Oksidaz Aktivitesi Üzerine Etkileri, EJONS Intenational Congress on Mathematic, Engineering and Natural Sciences – III, Nisan 21-22, 2018 Mardin/TÜRKİYE

2. Juglonun Salatalıkta (*Cucumis sativus)* Katalaz Enzimi Üzerine Etkileri, EJONS Intenational Congress on Mathematic, Engineering and Natural Sciences – III, Nisan 21-22, 2018 Mardin/TÜRKİYE

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