Understanding the physical and biological effects of ultrasound on *Pseudomonas aeruginosa* biofilms

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**UNDERSTANDING THE PHYSICAL AND BIOLOGICAL
EFFECTS OF ULTRASOUND ON *PSEUDOMONAS*
AERUGINOSA BIOFILMS**

LAKSHMI DEEPIKA BHARATULA

School of Chemical and Biomedical Engineering

2021

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EFFECTS OF ULTRASOUND ON *PSEUDOMONAS*
AERUGINOSA BIOFILMS**

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School of Chemical and Biomedical Engineering

A thesis submitted to the Nanyang Technological University

in partial fulfilment of the requirement for the degree of

Doctor of Philosophy

2021

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research, is free of plagiarised materials, and has not been submitted for a higher degree to any other University or Institution.

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Bharatula Lakshmi Deepika

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20/05/2021

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Date

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Authorship Attribution Statement

This thesis contains material from one paper published in the following peer-reviewed journal in which I am listed as an author.

Section 3.2.4, Chapter 4 and excerpts of Abstract are published as L.D. Bharatula, E. Marsili, and J.J. Kwan, **Impedimetric detection of *Pseudomonas aeruginosa* attachment on flexible ITO-coated polyethylene terephthalate substrates**. *Electrochim. Acta.* 332 (2020) DOI: <https://doi.org/10.1016/j.electacta.2019.135390>

The contributions of the co-authors are as follows:

- A/Prof Kwan provided the initial project direction and edited the manuscript drafts.
- I prepared the manuscript drafts. The manuscript was revised by A/Prof Kwan and A/Prof Marsili.
- I co-designed the study with A/Prof Marsili and A/Prof Kwan. I performed all the laboratory work at the School of Chemical and Biomedical Engineering and Singapore Centre for Environmental Life Sciences Engineering. I also analyzed the data.
- All microscopy, including sample preparation, was conducted by me in the Biofilm Imaging Centre, Singapore Centre for Environmental Life Sciences Engineering.
- All the electrochemical data was gathered by me in Singapore Centre for Environmental Life Sciences Engineering.
- A/Prof Marsili assisted in the interpretation of the electrochemical impedance spectroscopy data.

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- I co-designed the study with A/Prof Rice and A/Prof Kwan. I performed all the laboratory work at the School of Chemical and Biomedical Engineering and Singapore Centre for Environmental Life Sciences Engineering. I also analyzed the data.
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- A/Prof Marsili helped with the project direction and interpretation of the electrochemical impedance spectroscopy data.

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Bharatula Lakshmi Deepika

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Abstract

Chronic bacterial infections pose a huge threat to global health due to emergence of antimicrobial resistance. While bacterial colonies grow in either a planktonic state or within a microstructure known as a biofilm, the latter accounts for 65% of infections. Biofilms are characterized by the generation of extracellular polymer substrates (EPS) and presence of chemical and genetic heterogeneity. These factors participate in hindering the effect of antibacterial agents favoring the antimicrobial resistance. As resistant strains and persisters surpass novel chemical treatment methods, a potential approach to tackle this issue is mechanically stressing the biofilm to either disrupt the biofilm or enhance the transport of therapeutics to specific targets. Yet, investigations on the interactions between mechanical forces and biofilm are limited. This work proposes to use minimally invasive high intensity focused ultrasound (HIFU) to exert stress on *in vitro Pseudomonas aeruginosa* biofilms.

This project takes a step further from the current literature and stresses upon the need for studying the biological changes that mechanical stress may cause in biofilms. The possibilities, advantages and limitations of such studies are described. In future, this will allow the translation of ultrasound as treatment strategy to clinical research. Another novel aspect is use of well-defined electrochemical sensing system to study the microstructural effects of HIFU on biofilms. Use of this prominent bio-sensing technique will allow real time monitoring of the said effects.

Here, the effects of HIFU are characterized using conventional methods, i.e., confocal microscopy, crystal violet assay, and colony forming units. Additionally, an electrochemical based impedimetric sensing system was optimized to rapidly detect the changes in biofilm microstructure. To this effect, indium tin oxide coated polyethylene terephthalate (ITO:PET) was chosen as the biofilm growth substratum due to acoustic impedance match with

surrounding medium, optical transparency, and electrical conductivity. Optical characterization of biofilm growth on ITO:PET across four days showed a gradual switch from reversible attachment to biofilm maturation.

The biofilm growth was also characterized using electrochemical impedance spectroscopy (EIS). This is an efficient technique to rapidly detect the viability and metabolic activity of the biomass in a non-destructive and low cost setup. To understand the meaning of electrochemical response after HIFU treatment, the optimization was necessary. Since the response of weak electricigens such as *P. aeruginosa* on ITO:PET is not well understood, the EIS parameters for the system were optimized across four days of biofilm development. A change in the impedance modulus and phase angle was observed as the biofilm developed. Relaxation time analysis showed a dominant time constant at approximately 1 second and confirmed the validity of a two-time constant equivalent circuit model for the biofilm impedance. The interfacial resistance calculated from the equivalent circuit analysis showed a rapid drop after bacterial attachment whereas capacitance of the biofilm was masked by the capacitance of ITO:PET. The trends for interfacial resistance and capacitance were independent to the geometry of the ITO:PET working electrode. EIS across a broad range of potentials with and without inhibitors showed a marked difference between the interfacial resistance of viable and energy inhibited biofilms. Moreover, EIS of exopolysaccharide Δpsl mutant showed a substantial drop in current. Overall, the results indicated that EIS enabled the detection of biofilm formation across large surface areas as early as 24 hours for the weak electroactive species *P. aeruginosa* using a flexible polymeric substrate.

Next, the effects of acoustic exposure on biofilm were investigated using confocal microscopy, crystal violet assay, and electrochemical monitoring. Although ultrasound facilitates biofilm dispersal and may enhance the effectiveness of antimicrobial agents, the resulting biological response of bacteria within the biofilms remains poorly understood. To address this question,

we investigated the microstructural effects of *P. aeruginosa* biofilms exposed to high intensity focused ultrasound (HIFU) at different acoustic pressures and the subsequent biological response. Confocal microscopy images indicated a clear microstructural response at peak negative pressures equal to or greater than 3.5 MPa. In this pressure amplitude range (greater than 3.5 MPa), HIFU partially reduced the biomass of cells and eroded exopolysaccharides from the biofilm. These pressures also elicited a biological response; we observed an increase in a biomarker for biofilm development (cyclic-di-GMP) proportional to ultrasound-induced biofilm removal. The biological response was further evidenced by an increase in the relative abundance of cyclic-di-GMP overproducing variants present in the biofilm after exposure to HIFU. The results, therefore, suggest that both physical and biological effects of ultrasound on bacterial biofilms must be considered in future studies.

Furthermore, the findings revealed that cyclic-di-GMP overproducing mutant strains were more resilient to disruption from HIFU at these pressures, suggesting that a biofilm may develop resistance towards ultrasound. Therefore, successive ultrasound treatments were studied to investigate the potential for biofilm resistance to HIFU. We studied planktonic cultures grown from untreated and HIFU-treated samples and observed subsequent biofilm growth. After the first HIFU-treatment, we measured an increase in c-di-GMP activity. Further HIFU-treatment resulted in a weakening of the biofilm followed by a strengthening. However, after three HIFU treatments, HIFU was still able to mechanically remove the cells from the surface. Based on all the previous observations, a continuous and real-time monitoring system was designed to aid the future studies on the effects of HIFU on biofilms.

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1. Background and literature review

1.1 Introduction to biofilms

Bacterial communities in nature survive in two forms, either as free-swimming bacteria (i.e., in a planktonic state) or as biofilms [1]. Biofilms form nearly everywhere and contaminate both industrial and medically-relevant surfaces. Biofilms are microstructural communities of bacterial cells shielded by extracellular polymeric substances (EPS) consisting of proteins, extracellular deoxy ribonucleic acids (DNA), and polysaccharides [2].

Biofilms are relevant to wastewater treatment, in which they facilitate organic carbon removal. They also contaminate drinking water pipes and facilitate metal degradation via biofouling and microbially induced corrosion (MIC), respectively [3,4]. When formed in the body or on artificial implants, the biofilms withstand high doses of antimicrobial agents favouring the emergence of antibiotic tolerance and resistance [5,6]. Considering that biofilms are ubiquitous and affect various applications, the focus of this report will be on medical biofilms. In the coming sections, biofilm growth process, key regulatory mechanisms in the biofilm development and potential alternative therapeutic strategies are discussed.

Many bacteria form biofilms. Though much of our discussion relates to the different bacteria, our focus here will be on biofilms formed by *Pseudomonas aeruginosa*. These bacteria are considered a model biofilm forming microorganism in *in vitro* systems with an established genome database [7]. *P. aeruginosa* is a Gram- negative bacterium and one of the leading causes of nosocomial infections [8].

1.1.1 Biofilm growth and development

P. aeruginosa contributes to both surface (e.g., implants, contact lenses, and urinary catheters) and non-surface (e.g., lung infection in cystic fibrosis, chronic wounds, and chronic otitis

media) related biofilm growth (Figure 1.1) [9–14]. These species form communities rapidly and strengthen with time.

Figure 1.1: Device and tissue related medical biofilms. Recreated from reference [15].

The development of a biofilm is a multi-stage process starting from initial attachment to dispersal of biofilm cells (Figure 1.2a). The role of each stage has been summarized as follows:

1.1.1.1 Reversible attachment

In a nutrient rich environment, a conditioning layer comprising of organic and inorganic substances in the bulk medium such as proteins, polysaccharides, and salts is formed on the substratum [16]. The conditioning layer modifies the substratum and enables the attachment of bacteria. The planktonic bacteria attach either physically through van der Waals forces, steric interactions, or formation of electrostatic double layer, or through its motile appendages such as flagella and type IV pili [17]. While type IV pili is considered to be the primary contributor to this effect, recent investigations have shown that substratum properties influence the choice

of the bacterial appendage [18]. For example, flagella was revealed to participate in initial adherence to hydrophobic surfaces [19]. The base substratum roughness, temperature, pH, and available energy are other variables that affect the attachment process [16].

1.1.1.2 Irreversible attachment and maturation

After the initial adhesion to a substratum, few cells may revert to their original planktonic state. However, a population of cells will irreversibly adhere through strong physicochemical interactions to the surface including hydrophobic-hydrophilic interactions and oxidation-hydration reactions with the conditioning layer [20,21].

As bacterial cells multiply, micro-communities begin to colonize the surface, further developing into a mature biofilm. These biofilms typically present physical heterogeneity due to the EPS and water channels (which govern the circulation of nutrients within the biofilm and excretion of waste to the bulk phase) [22]. Oxygen, pH, and nutrient concentration gradients contribute to the chemical heterogeneity [5,23]. Furthermore, the primary carbon source also plays a major role on the structure formed [24]. For example, if citrate is used as the source for carbon, *P. aeruginosa* formed flat structures. In contrast, mushroom caps are formed only when glucose is substituted as the carbon source.

1.1.1.3 Dispersal

The final stage of the biofilm development is the transition of the biofilm cells to a planktonic phase or smaller microcolonies. Biofilm dispersal is either active or passive [25]. Active dispersal is self-initiated by the bacteria through signaling molecules. In contrast, passive dispersal arises due to externally applied stresses such as fluid shear forces, and abrasion from solid particles. Degradation of the biofilm matrix, cell division of cells weakly bound to the substratum, and production of surfactants (e.g., rhamnolipids in *P. aeruginosa*) are a few mechanisms governing active dispersal, which is often controlled by quorum sensing,

nucleotide, and small RNA regulatory pathways [22]. Moreover, *P. aeruginosa* was shown to naturally generate a dispersal agent identified as *cis*-2-decenoic acid [26]. The physicochemical properties of the surrounding medium have also been reported to influence the dispersal in biofilms. Nutrient excess or starvation plays a major role in the dispersal [27]. Contact with hydrogen peroxide and nitric oxide may also induce chemically induced dispersal in *P. aeruginosa* [28,29].

Figure 1.2: a: Stages of biofilm growth and development with representative scanning electron microscopy images - 1: Reversible attachment 2: Irreversible attachment 3: Maturation I 4: Maturation II, and 5: Dispersal. The scale bars represent 10 μm . Reprinted with permission from Springer Nature [30]. b: Schematic showing components of biofilm matrix. Reprinted with permission from Springer Nature [2].

1.1.2 Components of biofilm matrix

The generation of EPS matrix in addition to the chemical and genetic heterogeneity is the differentiating factor between the biofilms and planktonic bacteria. While cells form just 2-5% of the biofilm, the remaining structure comprises of EPS. Water is the largest constituent of the EPS matrix whereas, polysaccharides, proteins, DNA, and lipids form the rest (Figure 1.2b). Here, the function of each matrix component is briefly discussed.

1.1.2.1 Exopolysaccharides

P. aeruginosa biofilms typically consists of three exopolysaccharides namely, alginate, Pel, and Psl. Alginate is high molecular mass, uronic acids dominated exopolysaccharide formed by homopolymeric sequences of polymannuronate and heteropolymeric chains of guluronate and *O*-acetylated mannuronate residues [2]. Alginate is a characteristic feature of mucoid *P. aeruginosa* biofilms and is responsible for the structural integrity of the mature biofilms. Moreover, it also participates in the microcolony formation during the irreversible attachment phase. The alginate overproducing mucoid biofilms are typically found in cystic fibrosis patients' lungs [31].

Pel and Psl are predominant in non-mucoid strains where, alginate synthesis is not possible [31]. Pel and Psl are branched heteropolysaccharides where, Pel is rich in glucose and, Psl, is a pentasaccharide with repeating chains of mannose, glucose, and galactose [32]. Early studies on the role of exopolysaccharides in biofilm formation determined that Psl influenced the early biofilm development whereas, Pel was important during biofilm maturation [33,34]. Since, it has been found that all wild type strains may not synthesize both the polysaccharides thereby, the role may vary based on the *P. aeruginosa* strain. Psl was reported as a primary exopolysaccharide in PAO1 strains and participated in its biofilm development, whereas Pel was redundant (Figure 1.3) [35,36]. Interestingly, recent investigation revealed a role of exopolysaccharide cross-linking in the viscoelastic nature of the PAO1 biofilm matrix [37]. Through rheological behaviour determination, it was found that Psl actively favoured formation of stiffer/elastic biofilms during early biofilm growth. As the biofilm matured, a shift towards viscoelasticity was observed due to domination of Pel. In PA14 strains where Psl is not synthesized, Pel maintains the biofilm architecture and is involved in the pellicle formation at the air-liquid interface (Figure 1.3) [36,38].

Figure 1.3: Influence of Pel and Psl on biofilm formed by PAO1 and PA14 strains of *P.aeruginosa*.

The scale bars represent 50 μm . Reprinted with permission from John Wiley and Sons [39].

1.1.2.2 Extracellular proteins

Extracellular proteins (cell appendages, enzymes, surface proteins) play a vital role in the EPS by binding with the other matrix components thereby, promoting cell-cell and cell-matrix interactions. For example, type IV pili, a type of cell appendages were able to recognize and specifically bind to extracellular DNA [40]. Similarly, another class of cell appendages, cup fimbriae were involved in the adherence of cells to surfaces [41].

Furthermore, the LipA extracellular lipase was reported to interact with the alginate in the matrix via electrostatic interactions [42]. Another study revealed that an extracellular adhesin protein, CdrA crosslinked with Psl to reinforce the biofilm matrix [43]. Most recent evidence showed that this reinforcement of structural integrity was due to inhibition of the proteolysis during CdrA-Psl interactions [44]. Additionally, it was found that surface proteins, specifically lectins, presented a high affinity binding towards carbohydrates in the biofilm matrix. For

example, LecA and LecB bound to galactose and fucose respectively and were involved in biofilm formation [45,46]. Most recently, it was found that LecB also bound to the disaccharides of α -mannose in Psl to stabilize the biofilm structure [47]. Since, fluorescently labelled lectins have been actively used as indicators of the polysaccharides in the matrix [48].

1.1.2.3 Extracellular DNA

Reports on extracellular DNA have speculated that it influences the biofilm maturation and dispersal phases [2] and is a primary component of biofilms formed in lungs of cystic fibrosis patients [49].

Extracellular DNA was majorly present in the non-motile stalk subpopulations of the mushroom capped *P. aeruginosa* biofilms where cell-cell communication was predominant [50,51]. The aforementioned interactions with type IV pili (see section 1.1.2.2) [40] and the recently discovered cross-linking with Pel via ionic interactions [52] are said to be crucial in the stable formation of mushroom cap structures in glucose dominant *P. aeruginosa* biofilms [24]. Production of extracellular DNA-degrading enzyme, DNase I actively dispersed the biofilm (see section 1.1.1.3) [50].

Moreover, extracellular DNA readily bonded with aminoglycosides and antimicrobial-peptides thus, contributing to the antimicrobial activity of the biofilms [53].

1.1.2.4 Lipids

The well-studied lipids in *P. aeruginosa* biofilm matrix are rhamnolipids belonging to glycolipids family [54]. They were observed to double as surfactants in the matrix by modulating the swarming motility characteristics [55]. These glycolipids were reported to participate in the initial microcolony formation and biofilm dispersion in addition to maintaining the water channels [31,56]. Yet, further investigations are required to understand the role of matrix lipids in the biofilms.

1.1.3 Role of molecular signaling pathways in biofilm growth

In addition to matrix formation, molecular signaling is key to biofilm growth and development. Two molecular pathways namely, cyclic-di-GMP, a nucleotide based secondary messenger system, and quorum sensing, a cell-cell communication system, are discussed below.

1.1.3.1 Nucleotide signaling: Cyclic di-GMP

Cyclic di-GMP (c-di-GMP) is a vital nucleotide signaling molecule that regulates the planktonic to biofilm lifestyle transition and vice versa [57]. This switch is characterized by the formation of the c-di-GMP and its degradation, respectively. Therefore, the planktonic phase of bacteria has low c-di-GMP levels whereas, a mature biofilm has high c-di-GMP levels. During the transition from planktonic to biofilm state, c-di-GMP or cyclic dimeric guanosine 3',5'-monophosphate is synthesized by interactions between two molecules of guanosine-5'-triphosphate (GTP) and enzymes known as diguanylate cyclase (DGC) (Figure 1.4). The DGCs are characterized by GGDEF (Gly-Gly-Asp-Glu-Phe) active site motif [58].

Figure 1.4: Formation and degradation of c-di-GMP through DGC and PDE respectively. Reprinted with permission from American Society for Biochemistry and Molecular Biology [57].

During the dispersal phase of the biofilm, c-di-GMP degrades to form 5'-phosphoguanylyl-(3'-5')-guanosine (pGpG) and/or guanosine monophosphate (GMP) by interacting with phosphodiesterases (PDE) (Figure 1.4). The PDEs are characterized by HD-GYP and EAL motifs. The degradation may occur as follows: (i) PDE containing HD-GYP domain directly

hydrolyzes to form GMP, or (ii) PDE comprising of EAL domain breaks c-di-GMP to 5'-phosphoguanylyl-(3',5')-guanosine (pGpG) followed by formation of two GMP molecules [58]. There are 18 GGDEF-domain proteins, 5 EAL domain proteins, and 3 HD-GYP domain proteins in *P. aeruginosa* and each domain regulates different function in bacterial virulence and biofilm development [57]. Few functions of c-di-GMP during biofilm development are discussed below.

During the initial attachment, c-di-GMP controlled the flagellar motility by forming a complex with FleQ, a flagellar expression regulator and downregulated the genes necessary for flagellar assembly [59]. Another proposed mechanism to reduce flagellar mobility involved the interaction of YcgR (a protein with c-di-GMP receptor) with FliG (motor) and MotA (stator) of the flagella when sufficient c-di-GMP levels were reached [60]. Interestingly, planktonic *P. aeruginosa* was also reported to undergo bacterial mechanotransduction by increasing c-di-GMP levels [61].

During the interactions between bacteria and substratum by swarming motility, SadC and BifA were identified as the DGC-PDE pair that impacted the biofilm development [60]. Whereas, when twitching motility dominated the irreversible attachment was promoted by type IV pili, whose expression is partly controlled by c-di-GMP. Here, FimX was identified as the protein with both DGC and PDE domains that powered the twitching motility by assembling type IV pili [62]. Most recently, *P. aeruginosa* was observed to sense the change in substratum stiffness and regulate the c-di-GMP levels via *fleQ*, *oprF*, and *wspF* pathways [63].

To reiterate, following reversible and irreversible attachment to the surface, biofilm maturation occurred where EPS formation was a crucial event. High levels of c-di-GMP were present during maturation suggesting a correlation between c-di-GMP and EPS. At this stage, CdrA was identified as a protein that interacted with and increased the c-di-GMP levels. On deletion of *cdrA* gene, the biofilm growth was stunted (Figure 1.5a,b,d). Recent studies showing the

binding between CdrA and Psl confirmed that EPS regulation was dependent on c-di-GMP [43]. Furthermore, WspR-WspF, a DGC-PDE pair were identified as c-di-GMP regulator and controller of the EPS and biofilm formation. The mutations of this pathway are associated with small colony wrinkled morphology biofilm with high production of Pel and Psl (Figure 1.5c,d) [64].

Figure 1.5: Biofilm growth by PAO1 a: Wild type (20x Magnification) b: upon deletion of *cdrA* (20x Magnification) c: upon deletion of *wspF* (40x Magnification) d: upon deletion of *wspF* and expression of *cdrA* (40x Magnification). Modified and reprinted with permission from John Wiley and Sons [43].

The role of c-di-GMP in active dispersal was studied by adding dispersal agents to the biofilm microenvironment. The dispersal by sodium nitroprusside, a NO donor and glutamate activated PDEs such as DipA, RbdA, and MucR and lowered the c-di-GMP levels [65,66]. Another study showed that on variation in LapG protein, c-di-GMP associated *cdrA* removed the bacterial cells from substratum and subsequently enabled dispersal [67]. Furthermore, a recent

investigation has shown that the c-di-GMP concentrations in the dispersed cells from biofilms are capable of differentiating between active and passive dispersal phenomenon [68].

1.1.3.2 Quorum sensing

Quorum sensing is a cell-to-cell communication process in which bacteria regulate their genes to undergo various processes such as biofilm formation, virulence, and antimicrobial resistance [69]. The bacteria produced, secreted, and detected extracellular organic molecules called autoinducers to monitor their cell density [70]. No regulation of quorum sensing occurred at low cell density. Once the cell density reached a specific threshold (as detected by intrinsic bacterial receptors), the biofilm regulation genes regulated in synchronization. The concept is illustrated in figure 1.6.

Figure 1.6: Illustration of autoinducer based quorum sensing signaling pathway. Recreated from reference [71].

The quorum sensing system consisted of an inducer secretor (written with the suffix 'I') and receptor (written with suffix 'R') pair. The receptors received the secreted autoinducers only if the cell density was sufficiently high to form biofilms. For *P. aeruginosa*, the well understood quorum sensing signaling systems are LasI-LasR and RhII-RhlR systems [72].

Acyl homoserine lactones (AHL) are a major class of autoinducers in Gram- negative bacteria. N-(3-oxododecanoyl)-homoserine lactone (OdDHL) secreted by LasI and N-

butyrylhomoserine lactone (BHL) secreted by RhlI were the primary AHLs responsible for quorum sensing regulation in *P. aeruginosa* [73,74]. Upon binding with their autoinducers, the receptors LasR and RhlR formed complexes and bound to the target genes, activating transcriptional expressions. These activated genes regulated much of the behaviour of the bacteria and bacterial colony, such as the initiation of biofilm formation, dispersal, virulence, self-induced cell death, and colonization [72].

Recently, 2-heptyl 3-hydroxy-4-quinolone (PQS), an autoinducer belonging to quinolone family was discovered [75]. PQS regulated its own production by controlling the expression of PqsR [76]. The genes encoded by *pqsABCDE* in the system synthesized 4-hydroxy-2-heptylquinoline (HHQ) which in turn produced PQS by hydroxylation process [77]. Although studies have shown detection of reduced biofilm formation and decreased virulence factors (elastase, pyocyanin, and rhamnolipids) in absence of PQS signaling, the exact working mechanism of these systems are still in development [78,79]. It is evident from the vast discussions in the literature that quorum sensing and signaling is the foundation of biofilm virulence, and targeting these systems may become a major strategy for the treatment of biofilms

1.1.4 Antibiotics/antimicrobial agent resistance mechanisms

Currently there are many reports that have identified the tolerance to antibiotic/antimicrobial agents as a major challenge posed by bacterial biofilms[5,6,80]. Few of the mechanisms responsible for this effect are described as follows:

1.4.1 Slow penetration of the antibiotics

The first line of defense presented by the biofilms against the antibiotics is the biofilm matrix. Few *in vitro* antibiotic diffusion measurements have shown that the antibiotics readily penetrated the EPS, since it comprises of 90% water [81]. However, when they reacted with

the other constituents of EPS, the activity of the antibiotics was deactivated, in turn retarding the treatment altogether [80]. For example, positively charged class of antibiotics were immobilized when they interacted with the negatively charged exopolysaccharides [82].

In addition to the hindrance by the EPS, slow diffusion of antibiotics through the *P. aeruginosa* biofilm was also due to intrinsic resistance pathways [83]. The uptake of antibiotics in Gram-negative bacterium depended on the transport through the semi-permeable outer membrane. However, the permeability through the outer membrane of *P.aeruginosa* was two orders of magnitude less than other Gram-negative species, limiting the uptake of the antibiotics [84]. This reduced penetration, in synergy with other intrinsic, adaptive, and acquired resistance mechanisms enabled antibiotic resistance.

1.4.2 Altered microenvironment and slow growth rate

Biofilms are generally heterogeneous in structure (for reasons mentioned earlier). As a result, the interior layers of biofilms comprise of a mixture of slow growing and dormant bacteria, which are believed to be tolerant to antimicrobial treatment [5,85]. Furthermore, the heterogeneous microenvironment create regions of nutrient deprivation, pH differences, and anaerobic niches [80]. All of these allowed the bacteria to adapt to antimicrobial agents leading to an increasingly resilient biofilm.

1.4.3 Persister cells

Persister cells are a small subpopulation of the biofilm microcommunity that have entered a highly protective, slow growing state [86] . These persister cells remained unaffected by antibiotics. Reports have also indicated that in addition to antibiotics, persister cells were resistant to chemical disinfectants such as chlorine bleach and glutaraldehyde [85]. These cells are considered a major threat to treatments because even a small fraction may recolonize the site of the infection.

1.1.5 Current research on biofilm treatment

1.1.5.1 Problems with current antibiotic treatment

The aforementioned resistance mechanisms pose a critical challenge for biofilm treatment strategies. Unfortunately, current treatment strategies still depend on usage of antibiotics or antimicrobial agents for biofilm eradication in the following ways : (i) prophylaxis: to prevent infection in non-infected people (ii) pre-emptive treatment: when the infection initially colonizes, (iii) empiric treatment: use of broad range antibiotics due to the unawareness of the bacterial species infecting, and (iv) definitive treatment: when there is an exact diagnosis of the culture present in the infection [87]. However, these are short term treatments as increased dosages may lead to the resistant infections. Sometimes, the use of the broad spectrum antibiotics is not sufficient for the treatment and the last resort antibiotics with massive side effects, for example colistin (causing kidney toxicity), have to be revisited [88]. Therefore, there is a high necessity for either delivering these antibiotics to the infection site or novel strategies to combat biofilm infections. The research for alternative treatment strategies mainly focuses on either chemical approach or physical- mechanical approach or a combination of both.

1.1.5.2 Chemical approach towards biofilm treatment

The chemical approach aimed at the fabrication of compounds to inhibit the pathways that contribute to biofilm development. The most important and widely studied targeted pathways include c-di-GMP signaling, EPS synthesis, and quorum sensing [89,90]. Although this topic extends beyond the focus of this thesis, few examples are stated below for a brief understanding of the progress in this field.

The GGDEF and DGC domain-containing proteins were the targets to inhibit c-di-GMP activity. Small molecular compounds such as DI3, LP 3134, LP 3145, LP 4010, LP 1062, and Ebselen were shown to reduce the DGC activity of WspR and PelD pathway in *P. aeruginosa*

[91–93]. In regards to PDE inhibition, benzoisothiazolinone successfully inhibited RcoR, a PDE promoting bacterial virulence [94].

For EPS, all the three components: alginate, Pel, and Psl were considered targets to inhibiting molecules. Exopolysaccharide degrading enzyme, glycoside hydrolase has been reported to disrupt *P. aeruginosa* early biofilm and mixed species *S. aureus*/*P. aeruginosa* biofilm [95,96]. DNases such as human DNase I has shown promise to degrade the eDNA present in the EPS of cystic fibrosis sputum [97]. Most recently, thiol-benzo-triazolo-quinazolinone compounds HI9 and 925 hindered the alginate production in *P. aeruginosa* [98]. Moreover, several small molecules (I2, I7, I9, I10, I11, I12, and I13) were reported to have anti-biofilm activity and repressed the EPS (Pel/Psl) production [99].

In regards to quorum sensing targeting, both PQS inhibitors and AHL inhibitors have been investigated [90]. A benzamide-benzimidazole derivative, M64 has been identified to hinder the PQS signal in turn, affecting the production of virulence factors and biofilm development process [100]. Several extracts from natural systems for e.g., halogenated furanone compound from microalgae *Dilsea pulchra*, MomL from Gram-negative bacterium *Muricauda olearia*, and flavan-3-ol catechin, a flavonoid compound from the bark of *Combretum albiflorum* have the capability to interfere with the QS system thereby, affecting the biofilm maturity [101–103]. Furthermore, naturally occurring compounds such as curcumin in turmeric, ajoene in garlic, and gingerol in ginger were found to be potential quorum sensing inhibitors [104–106]. Sulphur containing compounds derived from food products such as iberin, cheirolin, iberberin, sulforaphane, and alyssin have also shown great promise in QS repression [107].

Several chemical reagents have shown potential to disperse the biofilms. Nitric oxide donors at low concentrations are the most well understood dispersal inducers [29]. Other molecules such as synthetic anti-biofilm peptides and diffusible signal factor molecules have shown efficiency in dispersing biofilms [108]. However, a tedious screening of thousands of

molecules is required to find an effective compound. Therefore, application of mechanical stimuli to biofilms is a preferable alternative [89]. The current research on mechanical stimuli is discussed in the next section.

1.1.5.3 Mechanical stimuli to combat biofilms

Mechanical treatment approach involves exposing a biofilm to either light, sound, electric field, or magnetic field to exacerbate a bactericidal effect. For instance, iron oxide nanoparticles, when triggered by magnetic field, effectively inactivated *P. aeruginosa* biofilm via hyperthermia mechanism [109,110]. Similarly, ultrasound assisted cavitation in presence of antibiotics and micro-particles was efficient in debriding as well as killing biofilms [111,112]. Despite the observed advantages, only few physical and mechanical approach have been explored. High-velocity micro sprays, voltage controlled electrical stimulation and magnetic field targeting (for implants), external interfacial tension, microbubble assisted ultrasound therapy, photodynamic therapy, and plasma treatment have been described to induce cell death and dispersion [113–117]. Due to the novelty of this approach, the mechanisms inducing the changes and the efficiency of these processes are not well understood. Moreover, the processes may cause damage to healthy tissues in real life applications. Hence, further study on the optimization to localize these effects is necessary.

In this report, we propose to use non-invasive therapeutic ultrasound as a potential biofilm combating mechanism to mechanically stress the biofilm microstructure to understand the effect of physical stress in bacteria. A brief introduction to therapeutic ultrasound and its effects on biological systems are discussed in the following section.

1.2 Therapeutic ultrasound as a treatment response

Sound is a mechanical wave characterized by locally changing particle density in a medium [118]. The wave alternates between compression (high density) and rarefaction (low density)

regions resulting in a local increase and decrease in pressure respectively as shown in figure 1.7.

Figure 1.7: Changing particle density (TOP) and its analogous sound wave (BOTTOM). Here, C = Compression, R= Rarefaction phase of the wave. Recreated from reference [118].

Generally, sound is classified by the transmission frequency relative to human hearing and broadly divided into: sonic (human hearing, 20 Hz to 20 kHz), infrasonic (below human hearing, <20 Hz), and ultrasonic (above human hearing, 20 kHz) regimes [119].

Ultrasound is perhaps best known for its diagnostic and therapeutic applications and is considered to be a non-invasive and cost effective. One of the most established application of ultrasound is diagnostic ultrasound operating at frequencies between 2 to 15 MHz [120]. Diagnostic ultrasound typically uses pulses of a longitudinal pressure wave which propagates through various tissues in the body. Scattered, attenuated, or reflected sound from tissue, bones, or any aberrant materials are processed to form an image [121]. Interestingly, the use of ultrasound for therapy predates diagnostic ultrasound, but only recently has garnered much interest. Therapeutic ultrasound operates at frequencies between 20 kHz to 5 MHz and exerts

localized therapy on the target area [122,123]. Broadly speaking, therapeutic ultrasound uses the thermal effects from acoustic absorption, mechanical effects from the acoustic waves, or a combination of the two to provide the associated therapy [124,125]. In this report, we focus our attention on therapeutic ultrasound, as we propose to use the phenomena associated with therapeutic ultrasound to enhance the treatment of medical biofilms formed in the body. The following sections detail the basic theory of ultrasound production and its effects on biological systems.

1.2.1 Generation of Ultrasound

Piezoelectric transducers (PZT) are comprised of specialized materials (e.g., crystals, polymers, ceramics, or composites) used to generate and record sound. These materials convert mechanical stress to an electric charge and vice versa [126]. Therefore, as an electric voltage is applied to the PZT, the material will deform in shape and apply a force to the surrounding medium. If the supplied voltage oscillates, this electrical wave is converted to a mechanical wave that will propagate through the medium. Here, the material properties of the propagation medium plays a particularly important role in the propagation of the acoustic wave. Ultrasound traveling through any material tends to lose acoustic energy (i.e., attenuate) to the medium [118]. This attenuation arises as a result of acoustic impedance (Z_A), which is defined as the ratio of acoustic pressure (P_A) and particle velocity (ϵ).

$$Z_A = \frac{P_A}{\epsilon}$$

This definition is simplified as a product of medium density (ρ) and velocity of sound in the medium (c) as seen.

$$Z_A = \rho c$$

The acoustic impedance is strongly dependent on transmission frequency and determines the amount of reflected and transmitted sound at boundaries [127]. As a result, air ($\rho = 1.2 \text{ kg/m}^3$, $c = 330 \text{ m/s}$) is not an ideal medium for ultrasound transmission due to the large acoustic impedance mismatch with most PZT. For this reason, water ($\rho = 1000 \text{ kg/m}^3$, $c = 1480 \text{ m/s}$) or ultrasound coupling gels (primarily made of water) are typically used during the application of therapeutic ultrasound.

Figure 1.8: Beam profile of focused ultrasound in axial (XY) and lateral (XZ) direction

Much like other types of waves, it is possible to focus ultrasound and avoid potential off-target side effects. This is often conducted by multi-element beamforming or geometric lensing [128,129]. Because of the scope of this report, we will focus our discussion on focused ultrasound transducers. Specifically, a spherical concave ultrasound transducer uses the

geometric lensing mechanism to focus at the centre of the radius of curvature of the sphere. These single element transducers typically have a fixed driving frequency and beam profile determined by the construction of the transducer [130]. For spherical transducers, the focus is not a point but rather a small oval shaped volume resembling a grain of rice (Figure 1.8) with plane of maximum intensity at the centre. The intensity in the focus (W/m^2) is defined as:

$$I = \frac{p^2}{2\rho c}$$

Where, ρ is the density of the medium and c is the speed of sound.

This plane with the geometric focus is known as the focal plane and determines the maximum width of focal volume in lateral axis. The axial resolution of the focal volume is known as focal zone and is bounded by a signal amplitude drop of 6 dB from the maximum amplitude.

1.2.2 Effects of therapeutic ultrasound

As mentioned earlier the effects of ultrasound are broadly classified into thermal and mechanical effects [120,131]. Both of these effects are influenced by the applied frequency, pressure amplitude, and exposure time.

1.2.2.1 Acoustically driven mechanical effects: Nonlinear acoustics

When the total acoustic pressure in a propagating wave is no longer small relative to the hydrostatic pressure, a distortion of the acoustic wave arises. Under such conditions, rarefactional part of the wave (negative pressure) is delayed while the compressional part of the wave (positive acoustic pressure) moves forward [132]. Here, the distortion increases with the propagation distance and further transforms into a shockwave. In this shockwave, the fundamental frequency (f) of the acoustic wave is accompanied by its harmonics (nf i.e., $2f$, $3f$ etc.) [133]. The formation of shockwaves may exert several changes in the target sample properties.

At high amplitudes of ultrasound (where non-linear wave propagation prevails), mechanical effects exclusively driven by acoustics may be observed. The acoustic driven effects are primarily acoustic radiation forces and acoustic streaming [123,124]. As an acoustic wave interacts with an object, the local pressure differences results in an apparent force (i.e., acoustic radiation force) acting onto the object. The acoustic radiation force is proportional to the pressure amplitude, size of the object, and the absorption coefficient of the object. When acoustic radiation forces are sufficiently large, acoustic streaming (or acoustophoresis) is observed [134]. Acoustic streaming arises from the ultrasound pushing the propagation medium which in turn impose stresses on the adjacent surfaces.

1.2.2.2 Absorption-driven thermal effects

As the ultrasound wave travels through the medium, part of the mechanical energy is absorbed and converted to thermal energy. From the perspective of the acoustics, absorption (or attenuation) is proportional to pressure amplitudes, frequency, and exposure duration [133]. The dependence of attenuation coefficient on ultrasound frequency is:

$$\mu = af^b$$

Where, μ = attenuation coefficient, f = frequency (Hz) and ‘a’ and ‘b’ = material specific constants.

Absorption is also dependent on the material properties of the medium. The loss of acoustic energy in the medium is often measured by the attenuation coefficient which is determined by the sum of the absorption coefficient and the scattering coefficient [131]. Therefore, acoustic intensity exponentially decays as a function of travel distance in a medium and attenuation coefficient μ .

$$I = I_0 e^{-\mu x}$$

Where, I = acoustic intensity, x = distance travelled, I_0 = initial acoustic intensity at the origin point. As energy is absorbed the local temperature begins to elevate.

1.2.2.3 Acoustic cavitation related mechanical effects

As established previously, acoustic waves propagate through an aqueous medium. The aqueous medium is reported to undergo energetic motions simulated by the ultrasonic field. Further, these simulations may form new cavities or nucleate pre-existing cavities [135]. Here, the cavity is defined as a voluminous bubble consisting of gas or vapour. The formation or nucleation of gas pockets occurs when the pressure amplitude crosses a certain threshold. Therefore, this phenomenon is a threshold driven process and is known as acoustic cavitation. For formation of new cavities, the forces exerted on the liquid required to initiate cavitation occur only at acoustic pressure amplitudes capable of tearing the liquid apart, i.e., at strengths equal to or greater than the tensile strength of the liquid [118]. Exogenous bubbles already present in the system may pulsate, grow, and cavitate in the bulk medium [136]. Such an exogenous bubble may also be gas trapped within nanoscale crevices [137]. The surfaces with the crevices may be (i) externally introduced or (ii) present in system due to inhomogeneity of the aqueous medium.

Cavitation is a complex process and is not limited to nucleating bubbles. The process described above is simply cavitation inception and the processes post this inception are classified into two categories: (i) non-inertial or stable cavitation and (ii) inertial cavitation (Figure 1.9). [118].

Non-inertial cavitation is characterized by small amplitude radial oscillations of the bubble in response to the acoustic pressure wave. The bubbles remain stable across multiple acoustic pressure cycles without collapse [138]. Here, the expansion of bubbles occur in the rarefaction phase whereas, contraction occur in the compression phase. Non-inertial cavitation results in

formation of microstreams thereby, exerting shear stress on the target surface [139]. Microstreaming is a result of non-inertial cavitation event where momentum transfer occurs from bubble to the surrounding medium via interfacial friction (Figure 1.10) [125,140].

Figure 1.9: Types of cavitation: a: Stable cavitation and b: Inertial cavitation. Recreated from reference [123].

Figure 1.10: Mechanical effects of cavitation. Modified and reprinted with permission from Elsevier [139].

In contrast, if the bubble radius expands beyond a certain threshold, the bubble will collapse due to the inertia of the surrounding water [118]. Inertial cavitation typically occurs when the sound wave enters the compressional phase. As a result, the bubble dynamics become unstable, and the behaviour of the bubble is dominated by the momentum of the fluid phase and not the acoustic field. These collapses are often characterized by the presence of a shockwave (with extremely high initial velocities), that forms during the collapse of the bubble (Figure 1.10) [141]. Here, the bubble collapse is extremely violent and results in supersonic bubble wall velocities [138]. The supersonic velocities are a result of compression of gas contents in the bubble leading to exertion of high pressure on the bubble wall [118]. Further, the transition to supersonic velocity generates pressure disturbances in the surrounding medium, thereby emitting a shockwave [125].

When bubbles undergoing inertial cavitation interact with a solid boundary, high-speed microjets are generated [124]. During the collapse, the rapidly accelerating medium deforms the bubbles into toroidal shape in turn, forming high velocity jets [125].

The transition to inertial cavitation is often defined as the acoustic pressure amplitude required to initiate the unstable bubble growth and subsequent collapse and is termed as inertial cavitation threshold. The threshold is dependent on hydrostatic pressure applied to the liquid, number and size of the contaminants/nucleation sites in medium, frequency, pressure amplitude, and pulse length of ultrasound, surface tension, temperature and viscoelasticity of the liquid [142,143]. Theoretically, the literature has defined the inertial cavitation threshold in multiple ways: (i) When the maximum bubble radius exceeds transition radius (i.e., peak radius above which energy dissipation decreases) and critical radius (i.e., peak radius above which inertial forces dominate), inertial cavitation occurs [144]; (ii) When temperatures of bubble reach 5000 K upon collapse, inertial cavitation occurs [145]; and (iii) In small bubbles where surface tension effects dominate, a critical radius value exceeds such that peak negative

pressure of surrounding liquid will cause unstable bubble growth (Blake threshold) [118]. In experimental conditions, inertial cavitation threshold is defined as a threshold where probability of inertial cavitation is 50% [146].

The binary classification of cavitation is simplistic. It does not always reflect the complex behaviour of bubbles in an acoustic field. For example, both inertial and non-inertial cavitation may occur simultaneously in an acoustic field. In most non-ideal scenarios, where both the types of cavitation are present, the dominating effect is determined by the power spectrum of the received signal. Here, the presence of harmonics, sub-harmonics, and hyper-harmonics indicate non-inertial cavitation whereas, broadband signal is typical for inertial cavitation [120].

1.2.2.4 Acoustic cavitation enhanced thermal heating

Acoustic cavitation results in heating. This mechanism is known as cavitation enhanced heating. Studies on tissue mimicking gel phantoms have shown two separate heating regimes in presence of bubbles: (i) a linear regime, where the acoustic intensity is linear at pressures before the threshold and (ii) a heating enhancement regime, where the temperature drastically rises by as high as 330% at the inertial cavitation threshold or cavitation nucleation in some cases [147]. Here the bubbles dissipate the acoustic energy into thermal energy as a result of absorbing broadband emissions from the inertial cavitation since the absorption of acoustic energy is a frequency dependent process [138]. Viscous heating as a result of applied shear on the bubble is another proposed mechanism for this effect [124]. Ultrasound frequency, concentration of bubbles, and short pulse excitation are key parameters for obtaining the cavitation driven heating effects.

Based on the understanding of fundamentals and mechanisms discussed above, therapeutic ultrasound has since been applied for the treatment of cancer, uterine fibroids, and neurological

disorders [123,131,148,149]. It has also been proposed for the enhancement of drug delivery, gene therapy, and improved sensitization of tumours to chemotherapeutics [120,138,150]. Moreover, it is under clinical trials for the treatment of essential tremors [151]. Most recently, therapeutic ultrasound has been explored as a treatment strategy for infections caused by planktonic bacteria or biofilms [112,122,152–154]. The current literature in the ultrasound-bacteria interactions is detailed in the following section.

1.2.3 Therapeutic ultrasound on bacteria/biofilms

The timeline of the development of ultrasound as a treatment modality on planktonic cultures and biofilms is discussed below.

1.2.3.1 Effect of ultrasound and antibiotics on planktonic cultures and biofilms

Early studies in this field aimed at investigating the effects of ultrasound on planktonic cultures. Low intensity ultrasound in combination with antibiotics on planktonic bacterial cultures showed higher killing of the cells as opposed to antibiotics treatment alone [155,156]. The influence of gentamicin upon planktonic suspensions of *P. aeruginosa* and *Escherichia coli* was studied with or without ultrasound driven at 67 kHz frequency and 0.3 W/cm² acoustic intensity (Figure 1.11a) [155]. A bactericidal activity (two-fold loss in bacterial colonies) against the planktonic suspensions was revealed when gentamicin combined with ultrasound. Similarly, when ultrasound (operating at 70 kHz and 2.2 W/cm²) was combined with erythromycin, the viability of *P. aeruginosa* planktonic cultures reduced by approximately two orders of magnitude [156]. Yet another study investigating the effect of ultrasound (70 kHz frequency and 4.7 W/cm²) and nitrocefin suggested that ultrasound increased the permeability of antibiotics by partially destroying the cell membrane of otherwise impermeable *P. aeruginosa* [157]. Therefore, a consistent synergistic bactericidal effect was observed in the previous investigations. In contrast, low intensity ultrasound treatment alone did not cause significant killing of the bacteria despite the ability to affect the cell membranes [152].

The complexity of the mechanisms increased when introduced to biofilm phase cells. Ultrasound exposure of one day old *P. aeruginosa* biofilms at 500 kHz and 10 mW/cm² demonstrated cell death when combined with gentamicin [158]. However, there was no change in the biofilm morphology when treated with ultrasound alone. In a similar study, *P. aeruginosa* biofilms grown on polyethylene substrata were exposed to a range of ultrasound frequencies: 70 kHz, 500 kHz, 2.25 MHz, and 10 MHz [159]. The acoustic intensity was maintained at 10 mW/cm² and the biofilms were supplemented with gentamicin. After 2 hours of ultrasound irradiation with gentamicin, a significant log reduction in cell viability was observed at all frequencies; effects at 70 kHz were most significant. The findings were concluded to be a result of enhanced antibiotic uptake due to acoustic streaming by stable cavitation [160]. The same was corroborated by studies on *P. aeruginosa* and *E. coli* biofilms at similar parameters [161]. When the influence of ultrasound exposure time (15, 30 and 45 min) at 70 kHz frequency and 1.5 W/cm² was investigated, the gentamicin uptake was increased in both *P. aeruginosa* and *E. coli* biofilms grown for 24 hours.

Although the aforementioned studies gave an insight into synergy of antibiotics and ultrasound on biofilms, they were primarily characterized by CFU. CFU studies gave an understanding of the changes in cell viability however, the biological mechanisms triggering the cell death were not explained. Most recently, a significant change in metabolic activity as assessed by XTT assay and biofilm morphology characterized by confocal microscopy were observed under ultrasound and antibiotic treatment conditions [162]. To observe the effects, a frequency ramp ranging between 450, 530, 610, 650, and 700 Hz was done for 2 min in presence of tobramycin. All frequencies resulted in the reduction of cell viability in the biofilms. Moreover, quorum sensing, motility, protein metabolism, EPS synthesis, and emergence of virulence factors in *P. aeruginosa* were among the genetic factors affected by low intensity ultrasound and antibiotic treatment [163]. In this study, the *P. aeruginosa* biofilms were irradiated with ultrasound at 50

kHz, 20 cycles and 250 Hz PRF; Gentamicin was used as the antibiotic. The susceptibility to gentamicin was increased by altering the expression profile of genes. Mechanical effects specifically cavitation was suggested to be the reason for the observed bio-effects.

At the low intensity, ultrasound caused minimal impact to bacteria microstructure, dispersal, and death [122]. Yet, on combination with free-floating antibiotics, the susceptibility to cell death increased. However, cavitation is a stochastic process and a control on cavitation or thermal effects is required to note enhanced and targeted treatment. To build upon these observations, the focus has since shifted to using cavitation agents such as microbubbles in synergy with ultrasound and antibiotics to achieve controlled release of antibiotics.

1.2.3.2 Ultrasound mediated microbubble destruction

Ultrasound mediated microbubble destruction (UMMD) used ultrasound intensity less than or equal to 1 W/cm^2 at frequencies varying from 80 kHz to 1 MHz in presence of microbubbles and antibiotics [153]. The studies were primarily performed on Gram- positive *Staphylococcus* species. The studies unanimously reported little to no effect of ultrasound or microbubbles alone on the bacterial biofilms [164–170]. However, in a combined effect of ultrasound with antibiotics and microbubbles, enhanced effect of the antibiotic delivery has been observed. In fact, the antibiotic uptake in ultrasound/microbubbles/antibiotics improved in comparison to the ultrasound/antibiotics treatment. The biological effects included downregulation of genes encoding principle EPS components, bacterial phagocytosis, reduction in biofilm thickness, damaged cell wall and protoplasts, and formation of micropores.

Similar to Gram-positive biofilm results, eradication of biofilms due to synergistic influence of microbubbles, antibiotics, and ultrasound was observed in Gram-negative biofilms. One such study evaluated the role of gentamicin, microbubbles, and ultrasound on *P. aeruginosa* biofilms [111]. The ultrasound exposure parameters were: 500 kHz driving frequency, 1.1 MPa

peak negative pressure, 16 cycles, 5 min exposure time and 1 kHz PRF. Here, the UMMD treatment significantly impacted the biofilm microstructure, i.e., biofilm biomass, thickness and surface roughness (Figure 1.11b). Interestingly, a reduction in carbon dioxide production indicative of biofilm respiration was also observed after UMMD treatment. Another study used the UMMD treatment on *Acinetobacter baumannii* biofilms [171]. Chitosan-modified polymyxin B-loaded liposomes and microbubbles were added to exposure chamber and ultrasound was irradiated for 5 minutes at 1 MHz central frequency and acoustic intensity of 3 W/cm². Here, the scanning electron microscopy images revealed presence of bacteria without a biofilm matrix indicating the disruption of morphology. Moreover, a reduction in colony counts and increased drug release kinetics suggested the anti-biofilm effects of UMMD treatment. The use of UMMD was extended to biofilms relevant in industrial applications. Mixed species biofilm collected from wastewater plant showed a loss in the EPS components namely polysaccharides, proteins, and lipids along with the loss in biomass due to generation of shock-waves and liquid jets [172]. The ultrasound pulses were triggered at 42 kHz driving frequency with 2 s on-time and 2 min off-time.

Although the literature often attributes cavitation as a primary participant in the UMMD related effects, a direct correlation between cavitation and biofilm eradication has been elucidated only recently. High-speed imaging captured the formation of liquid jets and subsequent collapse of the microbubble on the *Pseudomonas putida* biofilm surface at high pressure amplitude (~0.7 MPa; 1 MHz central frequency) leading to biofilm disruption [173]. In contrast no disturbance in the biofilm was observed at low pressure amplitudes (~0.1 MPa) and in absence of microbubbles. A recent study on ultrasound treatment (120 kHz, 0.44 MPa) of *in vitro* infective endocarditis model with *S. aureus* biofilms detailed the role of ultra-harmonic and broadband cavitation energy on the infected clots [174]. Here, stable cavitation was observed; broadband signal indicative of inertial cavitation was low. Given that the nature of cavitation is dependent

on the acoustic parameters, each study may indicate a different role of cavitation. Nonetheless, a change in biofilm morphology was consistently observed.

Despite the benefits of UMMD, it may suffer from challenges such as extended treatment time due to low sustenance time and large particle size of microbubbles [153,175]. As a result, other cavitation agents have been explored. For instance, gentamicin-loaded liposomes easily penetrated *Ralstonia insidiosa* biofilms under influence of pulsed ultrasound and showed decrease in viable bacteria due to acoustic streaming effect [176,177]. Here, a dual frequency approach was used to treat the biofilms. The liposomes penetrated the biofilms at 2.25 MHz, 10% duty cycle, 4.4 W/cm² acoustic intensity; the liposomes burst and delivered the drugs at 1.1 MHz, 10% duty cycle, 90 W/cm² acoustic intensity.

Use of acoustic nanodroplets (with a core of liquid perfluoropentane) driven by vaporization and cavitation increased vancomycin permeability and led to bactericidal effects in *S. aureus* biofilms [178]. Furthermore, a decrease in the metabolic cell activity as assessed by resazurin assay was observed indicating breakdown of cell wall. Here, an unfocused ultrasonic transducer was applied at 1.0 MHz frequency, 3.0 W/cm² acoustic intensity, 50% duty cycle and 5 min exposure time.

Recently, solid cavitation agents were investigated as an alternative to microbubbles due to smaller size and prolonged cavitation response [179]. Amphotericin B-loaded poly (lactic-co-glycolic acid) nanoparticles and ultrasound (42 kHz, 0.3 W/cm², 15 min) were investigated to treat fungal *Candida albicans* biofilms. The findings exhibited significant decay in cell viability and proteinase and phospholipase activity (indicative of biofilm virulence) in presence of ultrasound [180,181]. Formation of a patchy microstructure was observed under both *in vitro* and *in vivo* conditions. The effect of solid cavitation agents on bacterial biofilms are yet to be explored.

Figure 1.11: a: Ultrasound and gentamicin on planktonic *P.aeruginosa*. Reprinted with permission from America society of microbiology [155]. b: UMMD with gentamicin treatment of *P.aeruginosa* biofilm. The scale bar represents 10 μm. Reprinted with permission from Elsevier [111]. c: Effect of HIFU on *P.aeruginosa* biofilms. Reprinted with permission from Elsevier [182].

1.2.3.3 Influence of high intensity focused ultrasound on planktonic cultures and biofilms

Contrary to low intensity ultrasound, high frequency-high intensity ultrasound affect both planktonic bacteria and biofilms without external cavitation agents and antibiotics [122]. Application of high frequency (850 kHz) unfocused ultrasound to planktonic *Enterobacter aerogenes*, *Bacillus subtilis*, and *S. epidermidis* showed a correlation between bacterial survival and ultrasound power [183]. The microbial suspensions ($\sim 10^8$ cells/mL) were exposed to varying ultrasound power (varied from 10 to 62 W) where, a decrease in the cell viability was observed with increasing ultrasound power. At 62 W, *E. aerogenes*, *B. subtilis* and *S.*

epidermidis showed ~4.2, ~2.5 and ~4.4 fold log reductions after continuous exposure for 20 minutes. Moreover, a significant structural damage indicated the efficiency of ultrasound in bacterial inactivation. For *E. aerogenes* and *S. epidermidis*, the cells were misshaped and leaky. Yet, the cells did not break down. To the contrary, *B. subtilis* cells were broken down and most prone to acoustic driven damages.

Similar to unfocused ultrasound, focused ultrasound exhibited a steep decrease in the surviving bacteria within a minute of exposure time [184,185]. One study exposed suspensions of *E. faecalis* to HIFU at 0.25 MHz driving frequency 120 Vpp driving voltage and 30 to 120 s exposure time [184]. The CFU assay revealed no detectable CFU/mL after 60 and 120 s exposure indicating the bactericidal activity of HIFU without antibiotics.

The bacterial inactivation was also influenced by acoustic pressure and pulse length (or pulse repetition frequency (PRF)) [185]. Planktonic *E. coli* bacteria were exposed to ultrasound using 2 MHz focused transducer. A gradual decrease in the surviving CFU was observed as a function of exposure time (varied from 1 to 10 min) and acoustic pressure (varied from 2 to 10 MPa). Whereas, the bacterial inactivation was insensitive to variation of pulse length from 5 cycle-2 kHz PRF to 100 cycles-0.2 kHz PRF. Studies have suggested the role of sonochemical effects such as formation of hydroxyl radicals or mechanical effects such as inertial cavitation as the possible mechanisms for killing the planktonic bacteria [183,185].

Few reports also investigated the role of acoustically driven thermal effects on planktonic bacteria. A study focusing on thermal effects of HIFU showed a significant decrease in methicillin-resistant *S. aureus* (MRSA) bacterial load leading to reduction of abscess formed in a mouse model [186]. Here, HIFU transducer was operated at 3 MHz with four exposures of 9 s each followed by a 1 min off-time between each exposure. A moderate temperature (52.3°C) and high temperature (63.8°C) rise were tested on the MRSA loaded abscess. After 4 days of exposure, there was 1.45% and 94.5% decrease in the bacterial CFU after exposure to

moderate and high temperature conditions respectively. Most recently, a combination of mechanical and thermal effects were shown to irreversibly damage *Mycobacterium tuberculosis* cells after HIFU treatment in *in vitro* and *in vivo* setups [187]. The bacterial suspensions *in vitro* or bacteria loaded abscess *in vivo* were exposed to HIFU at 1 MHz driving frequency, 15 s treatment time and acoustic intensity of 3185 or 6369 W/cm². A decrease in CFU and morphological changes induced by cell wall damage were observed with increasing acoustic intensity. Moreover, the volume of bacteria loaded abscess reduced after HIFU exposure.

The production of high acoustic energy at a target area and interesting response in planktonic species meant HIFU may induce bactericidal and/or microstructural effects in biofilms. Yet, not many studies have extended the use of HIFU to biofilm treatment. Previously, HIFU (driving frequency = 1.1 MHz) was investigated to disrupt *E. coli* biofilms grown on 4-well chambered microscopic slides [188]. The biofilms were exposed to the following parameters: 9.1 ms pulses, 1000 Hz PRF and 30 sec total exposure time. The acoustic pressure amplitude was varied from 3.1 MPa to 7.6 MPa. HIFU exposure induced bacterial detachment from the substratum at high peak negative pressures (6.2 MPa and 7.6 MPa). Here, no countable CFU was observed at the higher pressures suggesting either cell death or cell dispersal. Yet, CFU as a characterization technique may be inefficient as the exact effects of HIFU on biofilm remained speculative.

A similar study investigated the influence of exposure time and burst period of 1.1 MHz HIFU transducer on microstructure of *P. aeruginosa* biofilms grown on graphite disks (Figure 1.11c) [182]. The biofilms were exposed to 13 MPa peak negative pressure and 20 cycle pulses whereas, the total exposure time was varied from 5 to 30 s and the burst period was varied from 1 ms to 12 ms. Unlike the previous report, epifluorescent microscope was used as a characterization tool where, live and dead bacterial cells were imaged. The findings revealed

the loss of both live and dead cells from the substratum at 30-s exposure and 6-ms pulse exposure. The results suggested that HIFU debrided the cells from the graphite disks however, cell death was not a primary consequence. Similarly, *E. faecalis* biofilms grown on petri dishes showed decrease in the biomass when exposed to HIFU at 0.25 MHz frequency [184]. Here, the exposure time was varied between 30, 60 and 120 s whereas, the peak negative pressure was set at 1 MPa. Confocal microscopy and electron microscopy showed a decrease in the thickness of the biofilm and disruption of the microstructure especially at 120 s exposure time. Furthermore, scanning electron microscopy presented disrupted cell walls and a patchy biofilm structure.

The three aforementioned studies of HIFU on biofilms ruled out thermal effect as a major consequence of the microstructural changes because, negligible temperature rise was observed. Therefore, mechanical effects, specifically cavitation, were considered to be the primary driving mechanism for the loss in biomass. The studies suggested a combined bactericidal and disruption effect however, the intracellular mechanisms responsible for these observations remained unclear.

In addition to cavitation driven HIFU, hyperthermia driven HIFU has also shown interesting results in biofilms. Notably, enhanced antimicrobial efficacy of drug-loaded, low temperature sensitive liposomes towards *S. aureus* biofilms was observed, resulting in cell death and matrix degradation [189,190]. Here, the low temperature sensitive liposomes loaded with ciprofloxacin were controlled by HIFU driven at 3.39 MHz, 7 W electric power and 30 min exposure time in both *in vitro* and *in vivo* conditions. The temperature was raised to 42°C and the ciprofloxacin release was examined by fluorescent spectrophotometer. Here, approximately 90% drug release was noted at increased temperature and a deformation of the matrix structure was observed from electron microscopy. The *in vivo* investigations indicated a decrease in the percentage of surviving cells under localized heating conditions.

Overall, HIFU disrupted both Gram- negative and Gram- positive biofilms and their planktonic counterparts via mechanical or thermal effects. Yet, complete (i.e., 100%) cell death was never achieved, which allows bacteria to recolonize and become more persistent. Moreover, it was unclear whether active or passive dispersal participated in the disruption of biofilms. For future considerations, investigating only microstructural changes is not enough to treat bacterial biofilms. A detailed study of the genetic makeup and the potential changes in their EPS and signaling pathways of the bacteria after HIFU treatment is necessary to comprehend the influence of HIFU on biofilms.

The aim of this thesis is to investigate the biological effects from biofilms exposed to ultrasound at different acoustic pressure amplitudes. In our study, we employ the acoustic exposure to the *in vitro* biofilm system. However, the detection of the biological effects of ultrasound on the biofilms needs a rapid detection strategy in addition to the present optically based gold standards that give the information about microstructure and microbial ecology. We introduce electrochemical techniques as a quick biofilm monitoring strategy after the acoustic exposure to understand the effects on the biofilm microstructure and activity.

1.3 Biofilm electrochemistry: Electrochemical impedance spectroscopy to monitor biofilm growth and development

Impedimetric sensing i.e., use of electrochemical impedance spectroscopy (EIS) has been applied to detecting various biological agents including antibody-antigen complex, aptamers, prokaryotic and eukaryotic cells, proteins, and enzymes [191]. Here, we focus our attention on prokaryotic cells or bacteria. The foundation of EIS as an analytical method for bacterial detection comes from microbially influenced corrosion, i.e., detection of corroded surfaces such as metal (e.g., steel) or non-metal (e.g., glass), colonized by microorganisms [192,193].

From there it has been extrapolated to applications in microbial electrochemical cells [194,195] and biosensors for bacterial attachment and biofilm detection.

In principle, biofilm grown on a conductive electrode forms a closed circuit in a three electrode system enabling current flow when a voltage is applied by a potentiostat [196]. The three electrodes comprise of (i) working electrode (WE): The potential of the WE is controlled by the potentiostat and biofilm grows on the WE; (ii) reference electrode (RE): Electrode with a known potential against which the potential of WE is measured; and (iii) counter electrode (CE): Acts as an electron sink or source depending on the nature of the WE. The electrochemical half-reaction occurs here and thereby, closes the circuit [197]. The schematic of the circuit is shown in figure 1.12.

Figure 1.12: Equivalent electronic circuit of a three-electrode based electrochemical cell. Here, i = current measured, V = voltage applied, WE = working electrode, CE = counter electrode, and RE = reference electrode. Recreated from reference [196].

The electrochemical activity at a WE is a multi-stage process involving (i) mass transport of oxidized species from bulk electrolyte, (ii) chemical reaction unrelated to redox reaction, for e.g., adsorption, desorption and decomposition, (iii) electron transfer at the electrode surface, and (iv) mass transport of reduced species to the bulk electrolyte [194]. Here, electron transfer by redox reactions is a crucial mechanism for the electrochemical sensing.

The microorganisms are capable of transporting electrons beyond their cell wall in order to respire [198]. While most microorganisms use soluble electron acceptors or donors, the ability of interacting with insoluble electron acceptor/donor has also been discovered [199]. This is known as extracellular electron transfer (EET) i.e., electron transfer when the acceptor/donor is incapable of entering the cells. The preliminary evidence of this mechanism was observed when iron-reducing microorganisms were able to transfer electrons to Fe (III) oxides [200]. Since, EET mechanisms for several bacterial species have been investigated.

1.3.1 Extracellular electron transfer mechanisms

Two types of EET processes have been understood namely, direct EET (DET) and mediated (or indirect) EET (MET) [201]. The types of EET are illustrated in figure 1.13.

Figure 1.13: Illustration of EET process: Direct electron transfer (LEFT) and Mediated electron transfer (RIGHT). Recreated and modified from reference [208].

DET occurs when the bacteria donates electrons directly to the electrode via periplasmic or outer membrane *c*-type cytochromes and conductive type IV pili (also referred to as bacterial

nanowires) [202,203]. This mechanism has been observed in *Geobacter sulfurreducens* and *Shewanella oneidensis* making them electrochemically active, biofilm forming species [204].

The EET mechanism in weak and non-electroactive microorganisms is explained by MET yet, strong electricigen *S. oneidensis* also undergoes MET [205]. Here, the electron transfer is accomplished through compounds known as electron shuttles which readily undergo redox reactions. These shuttles are either self-secreted or externally added. Some well-known self-secreted mediators include flavins in *S. oneidensis* and phenazines (e.g., pyocyanin) in *P. aeruginosa* [206,207].

Sometimes, MET is limited by weak signal response and photosensitivity of secreted compounds as found in case of pyocyanin [209]. Therefore, it is suggested to use exogenous redox mediators to amplify the signal response. In case of non-electroactive biofilms, the only signal response is from the exogenous mediators [210]. Few of these artificial redox mediators are ferrocene/ferrocenium couple, ferricyanide/ferrocyanide couple, and quinone derivatives [211–213]. Previously, supplementing *Enterococcus faecalis* biofilms with iron promoted the EET thereby, increasing the electroactivity [214]. Similarly, addition of quinone derivatives such as anthraquinone-2,6-disulfonic acid and 2-hydroxy-1,4-naphthoquinone (HNQ) to *Shewanella putrefaciens* and *E. coli* respectively enhanced the EET activity [204,215].

The EET is measured by either electrochemical impedance or voltammetric techniques. Though there is a great deal of literature on voltammetric techniques [216,217], the scope of this thesis will be within EIS. Thus, the following descriptions will pertain to the fundamentals of EIS.

1.3.2 Electrochemical impedance spectroscopy: Fundamentals

1.3.2.1 Fundamental Theory

EIS studies the impedance of the complex and dynamic systems. The concept of electrical impedance stems from a fundamental concept of ‘resistance’. Resistance is the ability of a circuit to oppose the flow of charges and is defined by Ohm’s law [218] as:

$$R = \frac{E}{I}$$

Where, R = ideal resistance (Ω), E= input voltage (V) and I = output current (A).

However, this concept is limited to a single circuit element, i.e., ideal resistor. An ideal resistor has various simplifying characteristics: (i) Ohm’s Law is followed at all voltage and current levels. (ii) The resistance value is independent of frequency, and (iii) alternating current (AC) voltage and current signals through a resistor are in phase with each other. However, real world scenarios contain circuit elements with a complex behaviour where, simplified properties of Ohm’s law may not be followed. The emergence of such complex behaviour leads to use of impedance, a general circuit parameter. Impedance extends resistance to AC systems where, a phase difference between input voltage and obtained current is apparent [219]. Electrochemical impedance is usually measured by applying a small sinusoidal AC potential to an electrochemical system and subsequently, measuring the current through the same. In a linear system, the output current response to sinusoidal potential remains sinusoid at the same frequency however, a significant phase shift is observed (Figure 1.14). Here, the phase shift in the current arises due to presence of circuit elements other than resistor in an electrochemical system. Such circuit elements include capacitance i.e., the capacity to store charges between conductors and inductance i.e., the induction of the voltages by changing magnetic fields of current.

Figure 1.14: Current response with a phase shift in an impedance system

In summary, impedance is a time domain function, dependent on the frequency of the applied sinusoidal input voltage [196,218]. Impedance is expressed as a complex number due to the presence of magnitude and phase angle. In electrochemical systems, the resistive current flow is defined by real impedance term due to the presence of only magnitude whereas, capacitance is represented in the form of imaginary impedance as it possesses both magnitude and phase angle [195]. Building upon Ohm's law, the impedance is represented as shown:

$$Z = \frac{E \sin(\omega t)}{I \sin(\omega t + \phi)}$$

Where, Z = Impedance (Ω), t = time (s), ω = radial frequency (rad/s), related to applied AC frequency ($= 2\pi f$) and ϕ = phase shift (rad) [219].

1.3.2.2 Graphical representation

EIS represents the impedance in two forms: (i) Nyquist plot and (ii) Bode plot (Figure 1.15) [219].

Figure 1.15: Illustration of a: Nyquist plot b: Bode -magnitude (BLACK) and Bode-phase (BLUE) plot

(i) *Nyquist plot*

The Nyquist plot (Figure 1.15a) in a complex plane represents the real impedance in x-axis and imaginary impedance in y-axis and each point corresponds to behaviour at one frequency.

As per the aforementioned definitions, the pure resistor detailed in real impedance is:

$$Z_r = R$$

Whereas, the imaginary impedance attributed to an ideal capacitor, becomes:

$$Z_i = \frac{1}{j\omega C}$$

Here, R = resistance (Ω), j = complex number, ω = angular frequency and C = capacitance

An important feature of Nyquist plot is that it moves from high frequency in the left to low frequency. The graph manifests in the form of a semicircle for a combination of resistance and ideal capacitance whereas, a depressed semicircle appears for non-ideal capacitance rising due to surface heterogeneity. However, one major limitation of Nyquist plot is that the frequency

information remains incomplete as one cannot simply attribute the frequency value to the obtained impedance by looking at the graph.

(ii) *Bode plot*

The limitation of the Nyquist plot is overcome by using a Bode representation. The Bode plot exclusively reveals the frequency behaviour for the obtained impedance in logarithmic scale at x-axis and the impedance magnitude (or modulus) and phase shift plotted in y-axis (Figure 1.15b).

The impedance magnitude represents the modulus of real and imaginary impedance:

$$|Z| = \sqrt{Z_r^2 + Z_i^2}$$

And phase shift represents:

$$\phi = \tan^{-1}\left(\frac{Z_i}{Z_r}\right)$$

The phase angle in the plot is also a representation of an additional parameter i.e., time constant which is:

$$\tau = RC$$

Where, τ is inversely proportional to ω . The presence of one time constant is attributed to one EET process in the system. Multiple loops in the Bode-phase graphs are associated with multiple electrochemical kinetic behaviours. In principle, the 0° phase shift represents a pure resistive behaviour whereas, -90° phase shift manifests a pure capacitive situation. The phase shift at $0^\circ < \phi < -90^\circ$ is associated with a non-ideal capacitance behaviour comparable to depressed semicircles in Nyquist plot.

1.3.2.3 Equivalent circuits and analysis

The obtained impedance data from Nyquist and Bode plots is commonly quantified by fitting to an equivalent circuit model. Though the circuit is chosen on the condition of best fit, the physicochemical understanding of the system is the prime motive for the circuit fitting analysis. Here, the circuit elements are common electrical elements like resistors and capacitors connected in parallel or series.

The most common and simplest equivalent circuit is the Randles' equivalent circuit (Figure 1.16) which summarizes the activity in bulk solution and at the electrode electrolyte interface [196].

Figure 1.16: Randles' equivalent circuit. Here, C_{dl} = double layer capacitance, R_{ct} = charge transfer resistance, Z_w = Warburg diffusion element, and R_s = solution resistance. Recreated from reference [219].

The equivalent circuits in a microbial electrochemical system are an extension of the Randles' circuit. The physical meaning of each element is as follows:

- (i) *Solution or electrolyte resistance (R_s)*

The high frequency and 0° phase shift component of the circuit corresponds to a pure resistive bulk solution or electrolyte behaviour. The conduction in the bulk solution is typically ionic where, a condition of electro-neutrality prevails [219]. The resistance of this bulk solution typically depends on the ionic concentration, type of ions, temperature, and area of the cell

containing the solution [195]. In a microbial system, the bacterial culture in the electrolyte also contributed to the solution resistance [220,221].

(ii) *Charge transfer resistance (R_{ct})*

The charge transfer resistance is influenced by the low frequency charge transfer kinetics due to the continuous electrochemical reactions at the electrode-electrolyte interface [219]. Here, the electrochemical reactions correspond to redox reactions, adsorption/desorption, and mass transport activity. The activation energy and the electrode-electrolyte distance also impact the charge transfer resistance. The current due this transfer is termed as Faradaic current which is the rate of charge transfer to the electrode. Few well studied modes of electronic conduction mechanisms in bio-electrochemical systems are: quantum mechanical electron tunneling and electron hopping through the electron shuttles [197].

During biofilm formation on electrodes, charge transfer resistance often becomes a marker of the monitoring due to increasing redox interactions between the electrode and the biofilm [222–226].

(iii) *Double layer capacitance (C_{dl}) or constant phase element*

In a solution, an electric double layer exists at the electrode-electrolyte interface. The double layer arises when the ionic and charged species in bulk transfer and electrostatically attract (or repel) to the interface [219]. The charges are separated by an insulating layer of few nanometres analogous to a capacitor.

As the name suggests, the double layer comprises of capacitance from two charged layers in the electrolyte namely: Helmholtz (or stern) layer and diffuse layer [219]. The strong electrostatically attracted species in the solution reside in the Helmholtz layer closer to the electrode whereas, the diffuse layer is a loosely attached ionic layer. The capacitance of

Helmholtz layer is considered to be $10\text{--}60\text{ }\mu\text{F}/\text{cm}^2$ irrespective of the medium whereas, diffuse layer changes depending on the electrolyte concentration and surface characteristics [196].

In experimental conditions, an ideal capacitance never exists due electrode surface inhomogeneity, applied voltage, and current distribution. Therefore, a constant phase element (CPE) is commonly used to represent non-ideal condition. In particular, CPE becomes more pronounced when the processes such as electrode porosity, electrode homogeneity, surface roughness, uniformity of adsorption on electrode, and grain boundaries on the electrode surfaces are affected [219,227]. In a biofilm, the capacitance or CPE is an indicator of biofilm thickness and the surface coverage on an electrode [227–233].

(iv) *Warburg diffusion element*

In non-ideal experimental conditions, impedance contribution due to diffusion may be observed. Warburg element explains the diffusional behaviour in equivalent circuits. This element is attributed to the migration or diffusion of electroactive species towards the electrode and discharge [219]. Warburg diffusion element defines the mass diffusional characteristics of charges such as diffusion coefficient and diffusion length [234]. On a Nyquist plot the diffusion element manifests as a diagonal line with a slope of 45° following the semi-circular behaviour whereas, on a Bode plot, a phase shift of 45° is observed [195]. However, it is difficult to recognize its existence in a fitting model as it is associated with charge transfer resistance and both elements appear at low frequencies.

1.3.3 EIS for Gram-negative bacteria/biofilm detection

1.3.3.1 EIS on electroactive biofilms

Biofilms formed by electroactive bacteria (for e.g., *G. sulfurreducens* and *S. oneidensis*) have been the source of understanding electrochemical mechanisms in bacterial sensing due to their capability of undergoing DET [227].

Previously, a comparison of blank and biofilm-colonized glassy carbon electrode was conducted using a standard three-electrode setup where, KCl calomel electrode and platinum wire acted as reference and counter electrodes respectively [235]. A decrease in the impedance magnitude and shift to non-ideal capacitive characteristics were observed after colonization of glassy carbon electrode by thin, 3-day *G. sulfurreducens* biofilms (Figure 1.17). Here, the biofilm was able to influence the charge transfer and capacitance behaviour of glassy carbon electrode at low frequencies (between 1 to 10 Hz). The change was supposedly a consequence of DET process between biofilm and electrode.

Figure 1.17: Bode plot of blank electrode vs 72 hour grown *G. sulfurreducens* biofilm. The circles represent left axis plot (mod Z) and the squares represent right axis plot (phase angle). Reprinted with permission from American society of microbiology [235].

The role of DET was corroborated by another report investigating the contribution of intracellular EET (DET) and extracellular EET (MET) response in electroactive bacteria [236]. The electroactive bacteria, *S. oneidensis* and *G. sulfurreducens* showed changes in the electrochemical signature at high frequency after initial bacterial attachment to the carbon electrode. Here, the biofilms were grown in a custom-made bio-reactor and EIS was recorded at -0.4 V bias potential (vs. Ag/AgCl). The report observed that DET was 95% and 80% of the

total impedance response in *S. oneidensis* and *G. sulfurreducens* biofilms respectively indicating the domination of DET in electroactive bacteria.

Another study investigating the continuous response during *G. sulfurreducens* biofilm development, adapted a flow-cell based monitoring setup to record EIS in the frequency ranging from 10 kHz to 10 mHz [237]. The findings revealed that conductance correlated linearly (positive slope) to biofilm growth indicating increased biofilm-electrode interactions. However, a saturating capacitance response was observed after an initial increase as the biofilm developed. These results showed the ability of EIS to temporally track and detect the biofilm development via conductance and capacitance parameters.

1.3.3.2 Monitoring growth, development and disruption of P. aeruginosa biofilms by EIS

Despite the EIS sensitivity towards electroactive species due to DET process and subsequent current production, various real life settings where biofilms form, consist of relatively weak electricigens that do not produce current. Such biofilms are a nuisance in industrial and biomedical settings and need to be rapidly detected. Therefore, an understanding of the electrochemical activity of weakly- or non-conducting electricigens is necessary [205].

Detection of *P. aeruginosa* especially during early bacterial adhesion had an advantage in electrochemical response over other species due to the production of phenazines, the endogenous redox mediator [207,238]. The electrochemical activity of phenazines was apparent after initial attachment of *P. aeruginosa* on gold-coated interdigitated electrode [221]. At 1 MHz frequency, there was a decrease in bulk solution resistance after one hour of inoculation; yet another drop after 8-10 hours of bacterial growth was observed. Moreover, the report observed step-wise increase in the double layer capacitance after the bacterial adhesion, in 100 Hz frequency regime corresponding to the solution-electrode interface. Another study on a similar electrode material i.e., gold-coated interdigitated silicon wafer showed decreased solution resistance at high frequency [231]. Yet, contrary to the previous report, this study

showed approximately 10% decrease in the double layer capacitance after an hour of *P. aeruginosa* inoculation at 100 Hz frequency. The decrease in the solution resistance in the aforementioned studies was probably due to the change in bacterial cell concentration in the bulk media when the migration towards the electrode occurred. The capacitance was suggested to depend on van der Waals interactions, electrostatic interactions, and Lewis acid-base interactions between solution and the electrode [239,240]. Since the growth medium was different in both the studies, the surface charge density probably varied and thus, the change in capacitance was expected. Overall, consideration of the bacterial strain, growth medium, electrode material, and their interactions are necessary for an understanding of mechanisms driving the electrochemical response.

Sometimes, the endogenous redox mediators may not be sufficient to drive the EET or the bacterial species may not produce redox mediators. For such studies, external redox mediators were often added to aid the EET. For example, addition of ferricyanide/ferrocyanide couple or HNQ to the electrolyte exhibited a change in the charge transfer characteristics at the *E. coli* coated electrode [215,241,242]. The aforementioned studies showed the competence of EIS to detect the adhesion of weakly electroactive bacteria at the electrode via changes in impedimetric parameters such as charge transfer resistance and double layer capacitance with or without redox probes. Therefore, monitoring growth and development of biofilms by EIS is a promising approach. As the focus of this thesis is on monitoring *P. aeruginosa* biofilms, the current literature in this field is discussed below.

Previously, the characterization of mature biofilms on stainless steel coupons in a standard three electrode setup exhibited an increase in the charge transfer resistance during the biofilm development and a dip after the biofilm dispersal process (Figure 1.18a) [243]. A saturated calomel electrode and a graphite electrode served as reference and counter electrode, respectively. Here, in presence of a redox probe, the bacterial cells were considered to be

insulating particles thereby, inhibiting the interactions between redox probe and the stainless steel coupon. The changes in the biofilm growth as observed by EIS were corroborated by confocal microscopy and electron microscopy. No suggestion on influence of capacitance was made. Growing static biofilms and transferring to a three-electrode electrochemical cell may affect the concentration of the phenazines in the system and therefore, external redox probes are necessary to drive the EET. However, a previous study suggested focusing on double layer capacitance as the electrochemical marker for biofilm detection in absence of redox probes [229]. Here, the EIS was recorded in three-electrode setup for pre-grown biofilms on platinum disc electrodes at various time periods. A platinum wire was the counter electrode whereas, Ag/AgCl acted as the reference electrode. A significant decrease in the double layer capacitance was observed as the growth time increased whereas, the control i.e., uncolonised electrode remained stable. It was suggested that extracellular bacterial components such as bacterial appendages (for e.g., flagella, pili, and fimbriae) and EPS may have influenced the diffuse layer as the biofilm matured. From the aforementioned studies, it was understood that, in presence of strong redox mediators, charge transfer resistance dominated the electrochemical response whereas, double layer capacitance dominated when a redox reaction was not possible. Yet, translating the biofilm characterization to a more dynamic flow cell setup without exogenous redox mediators, unveiled an influence of both charge transfer resistance and double layer capacitance on overall impedance [244]. Here, the biofilms were grown on gold/chromium/polypyrrole coated PET for 10 days. The results showed an increase in exponent of non-ideal capacitance (n) during early biofilm formation followed by gradual saturation response possibly due to interactions with exopolysaccharides and extracellular proteins. Moreover, a peak in charge transfer resistance during early stages of biofilm formation and a decrease during the stages of maturation was observed (Figure 1.18b). The change in resistance was probably due to the capability for self-generated phenazines to

undergo EET. Here, a consistent supply of phenazines may influence the resistance characteristics as evidenced by another study showing easier detection of *P. aeruginosa* in polymicrobial biofilm due to the production of pyocyanin, a type of phenazines [245].

Figure 1.18: a: Change in impedance response of *P. aeruginosa* coated stainless steel coupon. Reprinted with permission from Springer Nature [243]. b: Variation in charge transfer resistance and correlation with confocal microscopy as the biofilm matured. The scale bars represent 20 μm . Reprinted with permission from Elsevier [244]. c: Single frequency real time impedance response of *P. aeruginosa* biofilm growth A- blank electrode B- After bacterial inoculation and gradual biofilm growth C- After sodium azide insertion. Reprinted with permission from Elsevier [246].

Based on the understanding of the EIS characterization at a range of frequencies (as seen in the previous studies), a continuous monitoring method at a single frequency has also been adapted to attain faster detection of biofilms on electrodes. In addition to growth and development, the single frequency investigations were able to detect the removal or disruption of biofilms when

external biocides like chemical reagents or antibiotics were introduced. A consistent increase in impedance at a fixed single frequency (1300 Hz) was observed as the biofilm grown on gold sputtered PDMS matured (Figure 1.18c) [246]. On addition of sodium azide, a biocide that bound to the cytochrome oxidases and inhibited respiratory pathways, biofilm deactivation was observed in real time via decrease in impedance (Figure 1.18c).

Another study reported distinct impedance response at a single frequency (10 kHz) for wild type *P. aeruginosa* biofilms and its mutants corresponding to quorum sensing and EPS production [247]. This showed the sensitivity of EIS to differentiate between a standard bacterial growth and the one after mutations. Moreover, addition of L-arginine, a biofilm growth suppressor showed a significant difference in the electrochemical response after the biofilm disruption. The same study also reported the sensitivity of electrochemical measurements in tracking the changes in biofilm after addition of antibiotics such as ciprofloxacin, tobramycin and meropenem. Furthermore, EIS was able to monitor the degradation of the biofilm after its exposure to bacteriophages i.e., viruses that infect bacteria [248,249].

The current literature on EIS consistently showed a variation in electrochemical parameters after biofilm growth, development, disruption, and death. As a result, the research has been extrapolated to several real life applications including biofouling [250], water contamination studies [226,251], and medical microbiology [252,253]. Therefore, EIS was considered to be a promising technique to rapidly detect the changes in biofilms after ultrasound exposure.

1.4 Main objectives of the thesis

This thesis addresses the growth, development, and disruption of *P. aeruginosa* biofilms formed on indium tin oxide coated polyethylene terephthalate (ITO:PET) substrata. This interdisciplinary research is an amalgamation of microbiology, bioelectrochemistry, and therapeutic ultrasound. The specific objectives of the thesis are as follows:

Objective 1: To optimize and understand a rapid and non-invasive impedance spectroscopy technique to detect the biofilm development and subsequent effects of ultrasound.

Objective 2: To study the microstructural changes in the biofilms after ultrasound exposure using confocal microscopy, impedance spectroscopy and microbiological assays.

Objective 3: To investigate biological effects of ultrasound on biofilms by tracking the cyclic di-GMP response after ultrasound exposure.

Objective 4: Investigate the influence of successive ultrasound exposure on biofilm resistance

Objective 5: To incorporate understandings from previous objectives and develop a flow-setup to monitor the physical changes before and after ultrasound in real time.

1.5 Outline of the thesis

The growth and development of *P. aeruginosa* biofilms was monitored using optical methods and electrochemistry. Further, the interactions of biofilms and HIFU were assessed. Here, the microstructural and bio-effects were characterized. Finally, the real-time monitoring of biofilm-HIFU interactions using electrochemistry were explored. The chapter wise breakdown of the thesis is as follows:

Chapter 1 introduced the three main concepts explored in this thesis namely, biofilms, therapeutic ultrasound, and bioelectrochemistry with focus on development and disruption of *P. aeruginosa* species. The recent progress in treating *P. aeruginosa* biofilms with ultrasound and monitoring the biofilm development using EIS is shown. Finally, the objectives and overview of the thesis were discussed.

Chapter 2 discusses the experimental methodology used throughout the experiments. Majority of the experimental methods remain same throughout the thesis and hence, have been summarized in a single chapter.

Chapter 3 details the rationale behind the experimental design with focus on the choice of biofilm growth substratum. Furthermore, the biofilm development on the substratum of choice is explored using imaging and spectrophotometric techniques.

Chapter 4 builds upon the *P. aeruginosa* biofilm monitoring shown in chapter 3. Here, the characterization is done using EIS with and without external redox probe. This chapter discusses the impedance signature of biofilm across 4 days of growth and the role of viable cells and biofilm matrix in EIS detection. Moreover, the influence of electrode geometry (with and without biofilms) on the EIS measurement is explored.

Chapter 5 focuses on the microstructural and bio-effects of HIFU on *P. aeruginosa* biofilms. The microstructural effects are studied by staining the live-dead cells and EPS polysaccharides in the biofilms. The biological effect is studied by comparing the c-di-GMP concentration in biofilms with and without HIFU treatment. The possible mechanisms behind the observations were also explored.

Chapter 6 investigates the effect of multiple HIFU exposures on a biofilm. This is to understand whether the biofilms develop resistance to mechanical stimuli. The response of stronger biofilms formed by $\Delta wspF$ mutation were investigated. Further, the wild type biofilms were irradiated with HIFU three times to understand the resistance effects. Two protocols were explored.

Chapter 7 summarizes the final conclusions of the thesis and comments on the possible future directions.

2. Experimental methodology

2.1 Growth medium and bacterial culture preparation

A minimal growth medium known as ABTG was used in all the experiments [254]. Here, ‘A’ of the medium consisted of 15.1 mM ammonium sulphate ((NH₄)₂SO₄, Merck, Singapore), 33.7 mM sodium phosphate, dibasic (Na₂HPO₄.2H₂O, Calbiochem, Singapore), 22.0 mM potassium dihydrogen phosphate (KH₂PO₄, Merck, Singapore), and 0.05 mM sodium chloride (NaCl, Merck) whereas, ‘BT’ consisted of 1 mM magnesium chloride hexahydrate (MgCl₂.6H₂O, Merck, Singapore), 100 µM calcium chloride dihydrate (CaCl₂.2H₂O, Merck, Singapore), and 1 µM iron (III) chloride (FeCl₃, Sigma Aldrich, Singapore). Glucose (6.9 mM; C₆H₁₂O₆, VWR chemicals), acting as the carbon source formed the ‘G’ component of ABTG.

PAO1, a wild type and non-mucoid strain of *P. aeruginosa* and its mutants (Table 2.1) were used in the biofilm growth experiments. The overnight culture was prepared by scraping single colony bacteria from a tryptic soy agar plate and adding to 10 ml of Luria-Bertani (Becton Dickinson and Company, Sparks, USA) lennox broth. The culture was incubated for 16 h - 17 h at 37°C and 200 rpm shaking.

Strain and Thesis Section	Application	Ref.
PAO1 WT (Chapter 3: 3.2.1; 3.2.4 Chapter 4; Chapter 5: 5.2.2 Chapter 6: 6.2.3)	Analysis of biofilm viability using confocal microscopy (live/dead), crystal violet assay and electrochemistry for studies investigating the biofilm growth across 4 days and role of HIFU exposure at various acoustic pressures.	[255]
PAO1 Δpel (Chapter 4: 4.2.4)	<i>Pel</i> deletion mutant; Used to understand role of <i>pel</i> in impedance response.	[256]
PAO1 Δpsl (Chapter 4: 4.2.4)	<i>Psl</i> deletion mutant; Used to understand role of <i>psl</i> in impedance response.	[256]

PAO1 <i>PcdrA::Gfp</i> (ASV) (Chapter 5; Chapter 6: 6.2.2)	Mutant where <i>cdrA</i> gene is fused with unstable Gfp plasmid; (i) Used for c-di-GMP quantification in biofilms after HIFU exposure (ii) Used for biomass determination in EPS staining study	[257]
PAO1 $\Delta wspF$ <i>PcdrA::Gfp</i> (ASV) (Chapter 6: 6.2.1)	Mutant overproducing c-di-GMP; Used to study the role of c-di-GMP in biofilm after HIFU-treatment	[257]

Table 2.1: Various *Pseudomonas aeruginosa* strains used in the investigations

2.2 Biofilm growth using batch culture method

The biofilm growth method based on a previous report was adapted with slight modifications [29]. For the work described in section 3.2.1, 20x20 mm Mylar (Thickness = 25 μm , Professional Plastics, Singapore) and ITO:PET (Thickness = 125 μm , surface resistivity = 60 $\Omega/\text{sq.}$, Sigma-Aldrich, Singapore) were used as biofilm growth substrata.

Majority of the experiments (section 3.2.4, chapter 4, chapter 5, section 6.2.1, and 6.2.2), utilized 15x15 mm ITO:PET sheets. In case of ITO:PET geometry investigations (section 4.2.5), 15x15 mm square sheet bent at the centre (ITO coating on the outer side), 20x11.25 mm rectangular sheet, and 25x9 mm strip bent as a cylinder were used in addition to the 15x15 mm square sheet. Note that for the cylinder, an extra 10 mm was added and stapled in order to connect each end of the strip. An illustration of these geometries is shown in Figure 2.1.

Figure 2.1: Schematic representation of various ITO:PET geometries used

ITO:PET sheets were sterilized using UV and 70% ethanol (v/v). Then, they were glued to the base of a petri dish with silicone sealant (Selleys, Singapore) and filled with 15 mL ABTG medium. Noted exceptions were growth on ITO:PET bent square and cylinder where 25 mL ABTG medium was used in order to allow full immersion. The bent-square sheets were glued to petri dishes using a rolled autoclave tape whereas for cylinder, the stapled excess was glued to the petri dish. Each petri dish consisted of two ITO:PET sheets.

PAO1 overnight culture (described in section 2.1) was diluted by 100 times and added to the dish resulting in an initial optical density (at 600 nm wavelength) of ~ 0.02 . The petri dish was further incubated at 37°C and 50 rpm shaking (Incubation Shaker: Infors HT – Ecotron, UK). The sheets were cleaned in 10 mL medium and added to fresh growth medium every 24 hours. For biofilm growth monitoring described in section 3.2.4 and chapter 4, the bacteria grown across 24 to 96 hours were analyzed. All the other studies used biofilms grown for 3 days. The schematic of growth process is illustrated in figure 2.2.

Figure 2.2: Biofilm growth: Batch culture method.

2.3 Characterizing biofilms using imaging techniques

2.3.1 Fluorescent microscopy

Three μL of SYTO 9 (Invitrogen, Singapore) was added to 1 mL DI water and vortexed to make a stock solution. The biofilms grown on the sheets were stained by addition of 8 μL of SYTO9 solution. This was followed by inverting the sheets on a glass coverslip. Two

dimensional snaps of the samples were imaged by fluorescent microscope (Axio Observer Z1 inverted widefield, Zeiss) at 20x magnification.

2.3.2 Confocal microscopy

The bacteria grown on ITO:PET sheets for 24-96 hours (section 3.2.4) were gently dipped in fresh ABTG medium to remove loosely attached cells followed by addition of 4 μ L live/dead stain (see section 2.3.3 for staining protocol). The samples were then inverted on a glass coverslip and imaged under CLSM. CLSM (Zeiss LSM 780, Germany) operated at 63x oil resolution and 700 master gain, imaged four separate locations for each sheet.

The effect of HIFU at various acoustic pressures (Chapter 4) was analyzed using CLSM operated at 20x magnification and 700 master gain. Five separate locations at the acoustic focus were imaged on each sheet. Externally added stains or bacterial mutant strains, described in the following sections were used to analyze different components of the biofilm. The excitation/emission wavelengths for all such components have been summarized in Table 2.2. The biofilm samples were placed on ice after HIFU exposure and before imaging in order to exclusively capture HIFU related changes.

Fluorophore	Target	Excitation/Emission
SYTO 9	Live DNA	485/498
PI	Dead DNA	585/617
FITC conjugated con-A	α - Polysaccharide	492/518
CWR	β - Polysaccharide	365/435
Gfp (in PAO1 $P_{cdrA} :: Gfp$)	<i>cdrA</i> promoter gene	488/510
Cfp (in PAO1 $P_{cdrA} :: Gfp$)	Bacterial cells	435/485

Table 2.2: Excitation/emission wavelengths of fluorophores imaged under CLSM

2.3.3 Biofilm live/dead cell staining

The viability of the bacterial growth was monitored using *Baclight* live/dead (SYTO9/PI) stain (Invitrogen, Singapore). For this study, the biofilm samples (PAO1 WT) were stained with 8 μL *Baclight* live/dead stain (SYTO 9 and propidium iodide mixed in 1:1 Ratio in DI water) and imaged with CLSM.

2.3.4 Biofilm exopolysaccharides staining

PAO1 $P_{cdrA}::Gfp$ mutant was used for these studies where, the *Cfp* label was used as bacterial cell marker. Stock solutions with 1 mg/mL fluorescein isothiocyanate (FITC) conjugated concanavalin-A (con-A) (from Canavalia ensiformis, Sigma Aldrich, Singapore) in DI water and calcofluor white stain (CWR) (Sigma Aldrich, Singapore) mixed with 10% KOH solution (in 1:1 ratio) were prepared. 4 μL of these stock solutions were added to the biofilms and allowed to stain for 30 min each. Then, the stained samples were imaged under CLSM. Here, con-A stained the α -polysaccharides whereas, CWR stained the β -polysaccharides.

2.3.5 Analysis of volumetric parameters of biofilm microstructure

MATLAB (Mathworks Inc.) was used to calculate the volumetric parameters as per previous report [258]. Image processing steps before the volumetric calculations comprised of (i) Splitting the channels into separate image stacks (ii) Otsu's image thresholding, and (iii) image filtering using 2-D median filtering (filter size = 5) [259,260]. For the snippets of MATLAB code see appendix.

The biovolume of the biofilm was calculated as follows:

$$\text{Biovolume } (\mu\text{m}^3) = \sum_{x=1}^{N_x} \sum_{y=1}^{N_y} \sum_{z=1}^{N_z} \text{Image Stack } (x,y,z) \times \text{voxel size}$$

The porosity of the biofilms was calculated as follows:

$$\text{Porosity} = \frac{\text{Total no. of void pixels}}{\text{Total no. of pixels}}$$

The run-length was the count of continuous biomass pixels in a given axis (XY; XZ; YZ). From these values, the aspect ratio was calculated in each axis as:

$$\text{Aspect Ratio} = \frac{\text{X Run-length}}{\text{Z Run-length}}$$

It is to be noted that for all quantification data, the obtained values were further normalized with respect to the untreated control (grown from same bacterial culture).

2.3.6 Image analysis of c-di-GMP levels per biomass

PAO1 $P_{cdrA} :: Gfp$ and PAO1 $\Delta wspF P_{cdrA} :: Gfp$ mutants were used to quantify the relative amounts of c-di-GMP in HIFU treated and untreated biofilms [257]. Following HIFU exposure, the amount of cell biomass (cyan fluorescent protein (Cfp)) and *cdrA* expression (green fluorescent protein (Gfp)), which directly correlated to the c-di-GMP levels, were imaged by CLSM.

The c-di-GMP per biovolume (Gfp/Cfp) analysis was performed by MATLAB (Mathworks Inc.). First, a binary mask was created from the Cfp channel by thresholding and filtering the image as described previously. Further this mask was multiplied with the 8-bit Gfp and Cfp channel separately. The ratio of the two images gave a ratiometric image. Further, a quantifiable value was obtained by dividing the sum of Gfp matrix with that of Cfp matrix. For the MATLAB codes see appendix.

2.4 Spectrophotometric analysis of biofilm: Crystal violet assay

The ITO:PET sheets with bacteria were washed with phosphate buffer saline (PBS) solution made of 137 mM NaCl, 2.7 mM KCl, 10 mM $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, and 2 mM KH_2PO_4 [4]. Further, 0.1% (v/v) crystal violet solution (Sigma Aldrich, Singapore) was added to the sheets and they

were incubated at 37°C for 15 min. Following extra cleaning steps using PBS solution, 2 mL of absolute ethanol (Merck, Singapore) was added to the cleaned samples. Then, the absorbance was measured using a microplate reader (TECAN M200, Switzerland) at 550 nm. This technique was used in section 3.2.4 to characterize biofilm growth across 4 days and in section 5.2.2 to study biofilm microstructure after HIFU exposure.

2.5 Biofilm electrochemistry setup and analysis

2.5.1 Electrochemical cell assembly

A VSP or VMP3 multi-channel potentiostat (Bio-logic, France) operated by EC-Lab software (Bio-logic, France) was used for all the experiments. A three-electrode setup was assembled for the electrochemical investigations.

Figure 2.3: Schematic representation of electrochemical three-electrode setup

Here, coiled titanium wire and Ag/AgCl acted as the counter and reference electrodes respectively. ITO:PET sheet (with or without bacteria) connected to a platinum sheet electrode holder (Latech, Singapore) acted as the working electrode. The whole setup was submerged in

10 mL ABTG medium in order to keep the cells viable throughout the experiment. An anaerobic environment was maintained at the headspace of these electrochemical cells by continuous flushing of nitrogen gas. Figure 2.3 shows the illustration of the setup.

Potassium ferricyanide, an exogenous redox mediator was investigated at three concentrations (1 mM, 5 mM, and 10 mM). The setup was used for results in section 3.2.3, Chapter 4, and section 5.2.2.

2.5.2 EIS and analysis

The EIS response was obtained at open circuit potential (OCP) in the frequency range: 100 kHz to 30 mHz. The signal was excited at 10 mV amplitude sinusoidal potential. For the signal analysis, distribution function of relaxation time (DFRT) and equivalent circuit fitting were used.

DFRT was analyzed to determine the time constants presented by the biofilm on ITO:PET. Impedance spectroscopy evolutionary programming software was used to extract DFRT [261,262]. Then, the impedance response was fitted to an equivalent circuit model which was chosen based on the number of time constants identified in DFRT plots.

The equivalent circuit fitting using Z-Fit feature in the EC-lab software provided values of interfacial resistance and constant phase element (CPE). Hsu-Mansfeld equation was used to calculate the effective capacitance (C_{eff}) [263] :

$$C_{eff} = \frac{(QR)^{1/\gamma}}{R}$$

Where,

$Q = CPE (Fs^{(\gamma-1)})$, $R = \text{Charge transfer resistance } (\Omega)$, $\gamma = \text{CPE coefficient}$

2.5.3 EIS mechanism investigation

To investigate the EIS sensing mechanism, role of energy inhibitor and EPS were studied. 72 hours old biofilms were examined for these studies. For electrochemical investigation, staircase potential EIS (SPEIS) was conducted in addition to EIS at OCP. For SPEIS, a bias potential from 50 mV to 500 mV was added to OCP with 50 mV step size.

For energy inhibition, sodium azide (NaN_3) and carbonyl cyanide m-chlorophenyl hydrazone (CCCP) were used. The treatment was done in ABT (growth medium without glucose) in all cases. For the NaN_3 study, the ABTG medium was replaced by ABT consisting of 50 mM NaN_3 (in 20% (v/v) DI water). The petri dish was incubated at 37°C and 50 rpm shaking for 4 hours. The control biofilm samples were treated with 20% (v/v) DI water and ABT. For CCCP experiments, the growth medium was replaced with 500 μM CCCP dissolved in 0.1% (v/v) DMSO and ABT. The samples were then incubated at 37°C and 50 rpm shaking for 1 hour. For control experiments, the 72 hours grown biofilm samples were treated with 0.1% DMSO in ABT. Post-treatment, the samples were washed in ABTG to remove planktonic and loosely attached cells and electrochemical characterization was performed in ABTG as described above.

For EPS studies, PAO1 WT, PAO1 Δpel and PAO1 Δpsl biofilms were grown and electrochemical response was investigated.

2.5.4 Cyclic voltammetry and analysis

Cyclic voltammetry (CV) sweeps were scanned at rates 10, 30, 50, 100, and 200 mV/s in potential range: -0.6 V to 1.2 V. The diffusion coefficient of the blank electrode and the subsequent active surface area for colonized electrodes was calculated by Randles-Sevcik equation [197]:

$$i_p = 268600 \times A \times C \times \sqrt{n} \times \sqrt{(D \nu)}$$

Where, i_p = peak current (Amp), A = active electrode area (cm^2), C = concentration of redox species (mol/cm^3), D = diffusion coefficient (cm^2/s), n = number of electrons transferred in the redox event and ϑ = scan rate (V/s)

The kinetics of the anodic (k_a) and cathodic (k_c) reactions were obtained using Laviron equation [264]:

$$k_a = \frac{(1-\tau)nFv_a}{RT}$$

$$k_c = \frac{\tau nFv_c}{RT}$$

Where, R = ideal gas constant, T = temperature, F = Faradays constant, n = number of electrons transferred in a redox event, v = scan rate and τ = transfer coefficient

Here, τ is an unknown value. To calculate this value, the plot of the peak potentials against logarithmic value of scan rate (also known as trumpet plot) was obtained. The slopes of the anodic and cathodic plots are related to τ as follows:

$$m_a = 2.3 \frac{RT}{(1-\tau)nF}$$

$$m_c = -2.3 \frac{RT}{\tau nF}$$

Where, m_c = slope of cathodic plot, m_a = slope of anodic plot

2.6 HIFU setup and analysis

2.6.1 HIFU setup and its alignment with PCD

A HIFU setup was used for the calibrations and to study the effect of acoustics on biofilms. The calibration studies were done in a degassed water tank (1 m x 0.5 m x 0.5 m) with the HIFU transducer (Driving frequency = 0.5 MHz, Sonic Concepts, Bothell, WA, USA). The

focal length and width of HIFU transducer were 21.42 mm and 3.02 mm respectively. First, the PCD and HIFU were aligned so that the focal length of the PCD (63.5 mm) overlapped with the geometric focus of HIFU (63.2 mm). The alignment studies used an assembly of a Delrin holder tautly holding a stainless steel wire with a metallic bead. The HIFU, driven by a pulser/receiver, was focused on the metallic bead set at a distance equal to its time of flight. The same was repeated for a 7.5 MHz passive cavitation detector (PCD, Olympus Industrial, MA, USA). The time of flight was calculated using the following equation.

$$\text{Time of flight (TOF)} = \frac{\text{focal length}}{\text{speed of sound in water}} \times 2$$

2.6.2 HIFU pressure distribution calibration

Figure 2.4: An illustration of hydrophone calibration setup and custom-made sample chamber

To compare the pressure distribution of Mylar and ITO:PET sheets (Chapter 3), a custom-made sample chamber (inspired by [265]) allowing free-field acoustic transmission was manufactured. Here, a sheet of Mylar or ITO:PET was sandwiched between two Delrin blocks. The sample chamber was placed in between the HIFU setup and a needle hydrophone (75µm diameter, Precision Acoustics, Dorset, UK).

To determine the peak negative acoustic pressures, the pressure distribution fields of the HIFU were mapped using the needle hydrophone coupled with a submersible amplifier and DC coupler. The received signal was amplified 25x and the output was collected on the oscilloscope (DSOX3032A, Keysight Technologies, Netherlands). The setup schematic is illustrated in Figure 2.4.

Two types of scans namely, planar scan and point scan were performed using Ultrasound Measurement System (UMS) software. The planar scans gave a 2 dimensional pressure distribution map in XY, YZ, and XZ planes (Only XY plane for the pressure distribution fields of Mylar, ITO:PET and coupling cone). The signal was triggered at 40 mVpp input voltage, 20 cycles, and burst period of 10 ms by a function generator (Keysight 33210A). Point scan was performed to determine the output peak-to-peak voltage (mVpp) with respect to input voltage ranging from 10mVpp to 100mVpp. The peak pressure at the varying voltage values was further calculated using the equation shown below.

$$\text{Pressure(MPa)} = \frac{\text{Output Voltage}}{2 \times \text{Hydrophone Sensitivity}}$$

The hydrophone sensitivity was computed by extrapolating the frequency (1-30 MHz) vs sensitivity characteristics given by Precision Acoustics. The calculated hydrophone sensitivity of 75 µm needle was 3.55 mV/MPa.

To calibrate the voltage-pressure for coupling cone setup (detailed in next section) for biofilm exposure (Chapter 5), the tip of the coupling cone was sealed with Mylar sheet and a needle hydrophone (0.2 mm diameter, Precision Acoustics, Dorset, UK) was aligned and the aforementioned process was repeated. For 0.2 mm needle hydrophone, the sensitivity was 55.6 mV/MPa. For, non-linearity studies, the acoustic wave at peak negative pressure ranging from 0.5 MPa to 5.5 MPa (as calculated from hydrophone calibration) was recorded. Here, 1 mm

diameter hydrophone (Onda Corporation, Sunnyvale, CA, USA) was used to study acoustic wave propagation for non-linearity determination.

2.6.3 Ultrasound setup: Exposing biofilms

A customized three-layer sample chamber was manufactured to house the pre-grown biofilm on ITO:PET sheets. The top layer was designed to hold the tip of the coupling cone (Sonic Concepts, Bothell, WA, USA) attached to the 0.5 MHz HIFU transducer. As per coupling cone design specifications, the focus of the transducer was 5 mm away from the cone. Therefore, a 5 mm Delrin block filled with ABTG medium formed the second layer of the sample chamber. The first and second layer were sealed with Mylar sheet. The biofilm sheet was sandwiched between the second layer and third layer in turn, sealing the entire sample chamber.

For the HIFU treatment of biofilms, a function generator triggered an electrical signal which was further amplified by high power RF amplifier (Electronics & Innovation, Rochester, NY, USA). This amplifier was coupled to the HIFU transducer using an impedance matching network (Sonic Concepts, Bothell, WA, USA). The amplified signal was converted to acoustic waves by the HIFU transducer and transmitted to the target biofilm area. The output acoustic emissions were received by a PCD and then collected by an oscilloscope or data acquisition board (DAQ) (PCI-5122, National Instruments, Texas, USA). The schematic of the setup is shown in Figure 2.5.

Figure 2.5: An illustration of HIFU setup to expose biofilms and custom-made sample chamber. The exposure parameters were: 10% duty cycle, 50,000 cycles and 3 min total exposure time. The peak negative pressure amplitude was the variable factor in the experiments, ranging from 0.5 MPa to 5.5 MPa. For each experiment round, an untreated biofilm acted as a control. The quantified values from CLSM, crystal violet assay and electrochemistry were normalized with respect to the control biofilm.

2.7 Colony morphology analysis

An area, representative of the acoustic focus in the samples exposed to HIFU and the untreated control sample was scraped and mixed in LB medium. One mL of this solution was extracted and serially diluted till 10^5 dilution was reached. Final volume of 100 μ L of each dilution was individually plated on LB agar plates and incubated at 30 °C for 24 hours. After incubation, the colony forming units (CFU) were quantified using the formula:

$$CFU = \frac{\text{No. of counted colonies}}{\text{Dilution factor} \times \text{droplet volume}}$$

Here, circular and wrinkled morphologies were counted separately. The percentage of CFU with wrinkled morphology was quantified as follows:

$$\% \text{ of wrinkled colonies} = \frac{\text{CFU of wrinkled morphology}}{\text{Total CFU}} \times 100$$

Furthermore, both the morphologies were imaged by light microscope (Carl Zeiss Primo star) at 4x magnification.

2.8 Ultrasound resistance study

2.8.1 Protocol 1

To investigate the role of successive ultrasound exposure on biofilm, a resistance study was conducted. One mL culture from the overnight culture mix was extracted and CFU procedure

was conducted to get an initial measure of cells/mL. Further, biofilms were grown for 72 hours from the same overnight culture (see section 2.1 and 2.2).

Figure 2.6: Experiment flow of resistance study 1. S = Untreated control U = HIFU treated

After growth and the HIFU experiment (see section 2.6.3), bacteria from acoustic focus were scraped and added to 10 mL LB medium. One mL was extracted from both treated and untreated biofilms for CFU and the remaining medium was incubated for 16 hours. Further, separate biofilms were grown using the two overnight cultures; HIFU experiment was conducted to study the colony variant growth in 4 different treatment conditions (Untreated – Untreated, Untreated – HIFU-treated, HIFU-treated – Untreated, and HIFU-treated – HIFU-treated). Here, untreated – HIFU-treated means that the biofilm was an untreated control in round 1 and the biofilm grown from this culture was treated with HIFU in round 2.

The process was further extended for another round with 8 different combinations of untreated and HIFU-treated samples. CFU of both circular and wrinkled morphologies was obtained after each treatment. The schematic of the procedure is shown in figure 2.6. Here, S = Sham or untreated sample U = HIFU-treated sample.

2.8.2 Protocol 2

This previous protocol was improved such that all serial ultrasound exposures are done on a single ITO:PET sheet as opposed to scraping the culture and growing a separate biofilm from it. For these experiments, the biofilm growth explained in section 2.2 was modified such that only one ITO:PET sample was placed in one petri dish. The biofilm was grown under the same conditions as described previously. After the biofilm growth for 72 hours, the sample was exposed to HIFU and placed in a new petri dish. Fresh ABTG medium was added and the sample was incubated for 24 hours. Then, the biofilm was exposed to ultrasound on the second day and the same process was continued for one more day.

Following 3 days of repeated exposures, the area, representative of the acoustic focus in the biofilm was scraped and mixed in 1 mL ABTG medium. Further CFU analysis was done as per the procedure described in section 2.7. An untreated control was simultaneously run. An independent replicate was also analyzed with CFU just after one exposure for comparative studies. A separate analysis of confocal microscopy and c-di-GMP concentration (see section 2.3.6) was also run on independent technical replicates. The schematic of the procedure is shown in figure 2.7.

Figure 2.7: Experiment flow of resistance study 2

2.9 Statistical analysis

One-way analysis of variance (ANOVA) followed by Tukey post-hoc test was used to calculate the statistical significance. A significance value below 0.05 was considered statistically significant. The statistics were calculated with respect to independent technical replicates (N). The plots have been shown as scatter plots and box plots with data point overlap. For box plots, the box ranged between 25th to 75th percentile and the whiskers indicated the standard deviation. The horizontal line through the box represented median whereas the square shape represented mean. Only non-significant (NS) values were indicated in the box plots.

3. Experimental design criteria and biofilm growth optimization

3.1 Introduction

Recently, therapeutic ultrasound as a biofilm treatment strategy, particularly for those growing in a medical environment has shown great promise [153,154]. Yet, this research remains in the initial stages and thus, the influence of ultrasound related effects on the biofilm microenvironment is unknown [122]. Investigating the underlying mechanisms governing the acoustic-biofilm interactions is necessary to drive future innovations and subsequent translation to clinical research.

The material properties of the biofilm growth substratum play a particularly important role in investigating such applications. Ultrasound traveling through any material tends to lose acoustic energy (i.e., attenuate) due to acoustic impedance mismatch leading to effects including but not limited to sound absorption, wave scattering due to material inhomogeneity and wavefront divergence [118,142]. Therefore, the choice of an appropriate biofilm growth substratum is extremely crucial in the *in vitro* experimental setup.

Moreover, bacteria are able to grow on a broad range of surfaces. Thus, any acoustically and optically transparent plastic was considered eligible. [266,267]. Furthermore, electrochemical techniques have recently shown promise in rapid biofilm detection on conductive surfaces [197,268]. Their low cost setups and the capability to translate into commercial biofilm sensors encouraged us to explore this technique [253]. Thus, an appropriate support structure must (i) allow biofilm growth, (ii) be electrically conductive, (iii) acoustically transparent, and (iv) optically transparent for characterization using electrochemistry and microscopy.

In this study, we investigated Mylar (biaxially oriented PET), which has been previously used in ultrasound therapy experiments [265] and an electrically conductive indium tin oxide (ITO) coated polyethylene terephthalate (PET). Biofilm growth, optical transparency, acoustic transparency, and electrical conductivity were studied for these materials. Subsequently, the biofilm growth and development across 4 days was studied on the material of interest.

3.2 Results and discussion

3.2.1 Mylar vs ITO:PET – Biofilm growth and optical transparency

Figure 3.1: Fluorescent images of *P. aeruginosa* biofilms grown for 72 hours on a: Mylar and b: ITO:PET. SYTO 9 (live cell stain) stained the biofilms

Figure 3.1 shows the fluorescent images of the biofilm grown on Mylar and ITO:PET after 72 hours. Here, biofilms on Mylar (Figure 3.1a) exhibited slow growth whereas, ITO:PET (Figure 3.1b) showed a relatively faster growth which made it a material of interest in our studies. A myriad of physicochemical, chemical, and topographic properties like surface charge, surface roughness, hydrophobicity, and stiffness may be responsible for the difference in the biofilm development on Mylar and ITO:PET [267,269]. However, the underlying mechanism for this adhesion difference is still unknown and further investigation, outside the scope of our work is needed to quantify the difference in mechanical properties of the two films.

The clarity in the fluorescent images confirmed the optical transparency of the Mylar and ITO:PET sheets.

3.2.2 Mylar vs ITO:PET – Acoustic wave transmission

Although ITO:PET exhibited relatively faster biofilm growth, it was necessary to characterize the acoustic beam formation through it in order to adapt the substratum in our experiments. As a result, the acoustic properties of ITO:PET in comparison to water and nearly acoustically transparent Mylar were determined.

Figure 3.2: Lateral beam profile of HIFU through a: Free-field b: Mylar c: ITO:PET

Figure 3.2a-c show the lateral plane beam propagation through water, Mylar, and ITO:PET respectively. For ITO:PET, we observed a decrease in the intensity at the focus, which was probably due to the difference in the thickness compared to Mylar [266]. Scattering (reflection, refraction) of the incident acoustic wave due to a variation in the material topography was another possible reason for this effect [142,270]. These results showed that ITO:PET albeit the signal attenuation, did not distort the beam profile. This allowed us to maintain a nearly acoustically transparent setup where, the response was purely from ultrasound related mechanical effects as opposed to compounded response from the reflections of the ITO:PET. Therefore, ITO:PET was eligible for acoustic experiments.

3.2.3 Mylar vs ITO:PET – Electrochemical Response

EIS measured the impedance of the Mylar and ITO:PET sheets across a range of frequencies. Figure 3.3 shows the representative Nyquist plot of the EIS. Both the real and imaginary components indicating the resistive and capacitive components respectively were extremely high for Mylar. This indicated that Mylar was not electrically conductive. Thus, Mylar was not

an ideal substratum for electrochemical sensing [271]. In contrast, ITO:PET showed lower impedance characteristics suggesting the conductive nature of the plastic due to the ITO coating [272].

Figure 3.3: Nyquist plot of Mylar and ITO:PET

Further, the conductive nature of the ITO:PET sheet allowed us to monitor the biofilm development on ITO:PET [268]. The detailed description of electrochemically tracking the biofilm growth is explained in chapter 4.

3.2.4 Monitoring biofilm growth using optical methods

As ITO:PET showed faster growth and electrically conductive characteristics, it was used for all the subsequent experiments. The biofilm growth and development on ITO:PET substrata across 4 days (Figure 3.4) was characterized using optical methods namely: CLSM and crystal violet assay. After 24 hours growth only individual cells were visible suggesting that the cells underwent initial attachment phase (Figure 3.4a). Figure 3.4b showed that by 48 hours, clusters consisting of both individual cells and microcolonies were initiated. Continued growth for 1 more day (Figure 3.4c) resulted in a mature biofilm. This was in agreement with previous observations [1]. After 96 hours, the biofilms were highly variable with increased cell death (Figure 3.4d).

Figure 3.4: Representative CLSM orthogonal cross- sections of biofilm growth on ITO:PET at a: 24 hours b: 48 hours c: 72 hours and d. 96 hours growth.

Biomass variation at various locations on the same sample indicated that heterogeneous biofilms were obtained. The microstructure presented as a combination of mushroom stalks and flat structures. To a certain degree, the inversion of biofilms on coverslip for imaging purposes might have resulted in flat structures. Moreover, previous reports have shown that the primary carbon source affects the structure of the biofilm. In case of glucose, mushroom structures are dominant [24]. However, biofilms on hydrophobic polymers previously resulted in the combination of mushroom and flat structures [273]. Evidently, ITO:PET substratum contributed to the biofilm structure.

Further, quantitative analysis (Figure 3.5a) of the CSLM images revealed that the biomass of live cells increased from $0.03 \pm 0.02 \mu\text{m}^3/\mu\text{m}^2$ at 24 hours to $0.75 \pm 0.07 \mu\text{m}^3/\mu\text{m}^2$ at 72 hours. Then, it decreased to $0.36 \pm 0.12 \mu\text{m}^3/\mu\text{m}^2$ after 96 hours. Comparatively, the biomass of dead

cells increased until 72 hours and stabilized at 96 hours. These values agreed with the crystal violet assay results (Figure 3.5b). Crystal violet assay showed that the absorbance corresponding to the biofilm biomass increased until 72 hours and then decreased at 96 hours. Only statistically insignificant values were marked in the plots.

Figure 3.5: Live/dead biomass calculated by COMSTAT 2 (N=3, Mean $\pm$ SD). b: Crystal violet absorbance at 550 nm (N = 8, Mean $\pm$ SD), NS: non-significant.

According to the biofilm growth cycle discussed in section 1.1, after 24 hours growth, the cells initiated adhesion to the substratum as only individual cells are observed in CLSM [1]. Further, the cells started microcolony formation after growing for 48 hours. By 72 hours of growth, the biofilm entered maturation stage and increased in thickness by producing EPS. This was confirmed from crystal violet results which accounted for the whole biomass in the system including live cells, dead cells, and EPS; the significant increase in absorbance from 48 hours to 72 hours indicated increased biomass and EPS formation [274]. However, 96 hours growth noticeably showed a batch to batch variation in the system as the absorbance of crystal violet increased for few batches whereas, decreased for others compared to 72 hours growth. The observed decrease is presumably due to the high concentration of dead cells at 96 hours and/or dispersal (either self-induced or induced by the environmental cues) [22].

3.3 Conclusion

After considering factors such as biofilm growth, acoustic pressure distribution, and electrochemical characteristics, ITO:PET was selected for biofilm growth substratum for the subsequent experiments. Briefly, *P. aeruginosa* was unable to attach rapidly and form biofilms on Mylar, a widely known acoustically transparent material. As a result, ITO:PET belonging to the same family of polymers as Mylar was selected as the biofilm substratum, though there was some attenuation of ultrasound signal. Yet, the HIFU beam profile was not distorted from the presence of the ITO:PET sheet. Moreover, the electrically conductive property of ITO:PET made it an excellent candidate for biofilm sensing by EIS. Further probing into the biofilm development on ITO:PET by confocal image analysis and crystal violet assays showed a near-complete coverage of the substrate after 72 hours of growth.

4. Impedimetric sensing of *Pseudomonas aeruginosa* biofilm growth on flexible ITO:PET surfaces

4.1 Introduction

Current biofilm monitoring strategies rely on optical techniques such as microscopy, spectrophotometry, flow cytometry, and fluorescence *in situ* hybridization [274–277]. Although these techniques provide information on bacterial concentration, biofilm microstructure, and biofilm microbial ecology, they are time-consuming, expensive, and not suitable for rapid, *in situ* biofilm detection over large surface areas. Recently, electrochemical impedance spectroscopy (EIS) has been used to monitor biofilm development *in situ* [222,253,268,278]. The technique has garnered attention due to the low-cost setup and the ability to rapidly monitor large surface areas pertinent to industrial and medical applications. Thorough analysis of EIS results recorded at a range of frequencies and potentials provides information on the extracellular electron transfer (EET) between the electrolyte and an electrode in the presence of a biofilm interface. [201,227].

EET is typically measured for highly electroactive microorganisms such as *Geobacter sulfurreducens* and *Shewanella oneidensis*, which give a strong signal through direct electron transfer via transmembrane cytochromes and conductive pili or via secreted redox mediators (e.g., flavins for *S. oneidensis*) [201–203,206]. Whereas, weak electroactive biofilms such as *Pseudomonas aeruginosa* undergo EET via redox probe (self-secreted or exogenous)[205]. Furthermore, EET in EIS specifically gives information on the charge/electron transfer, capacitance, and diffusion limitations. Charge transfer corresponds to the redox reactions at the electrode-biofilm interface. Biofilm capacitance is primarily associated with the thickness and the surface coverage of the biofilm attached [227–229]. Additionally, the electrode properties such as porosity, surface roughness, surface area, and atomic-scale heterogeneity affect the

capacitance measurement [219,227]. Moreover, mass diffusional characteristics of charges from bulk to electrode specifically, diffusion coefficient and diffusion length are determined by diffusion element [234].

Most studies tend to investigate EIS behaviour on ideal, rigid substrata such as carbon, platinum, and gold [229,245,246]. These substrata have large electrical conductivity and smooth surfaces, which allows for better EIS characteristics and thus, higher sensitivity for biofilm monitoring compared to polymeric substrata. Yet for many applications, biofilms may contaminate polymeric surfaces across large surface areas ($> 200 \text{ mm}^2$), which may assume a number of different geometries. Polymeric substrata such as polyethylene terephthalate (PET) are of particular interest due to their use in food packaging, bottling, medical device packaging, and artificial implants [275,279,280]. PET's conductive counterpart, ITO:PET has also been actively researched for applications in biomedical devices and flexible optoelectronics [281,282]. These polymer films are prone to contamination from biofilm-forming bacteria, which cause biofouling in packaging industries [3] and pose serious risks in biomedical settings [5,6]. Few reports to date have utilized EIS to study the adhesion of bacteria and biofilm formation on ITO-based substrata [223,240]. Additionally, these studies were performed on glass-based substrata and were limited to initial bacterial adhesion.

Despite the advantages of EIS, calculations of the reaction kinetics, diffusion coefficient of redox probe and active surface area of the electrode are complicated. Relatively, cyclic voltammetry is a straightforward technique for the determination of such characteristics [194,283]. However, cyclic voltammetry does not differentiate non-faradaic and faradaic processes as well as EIS [216].

In the present study, EIS of *P. aeruginosa* biofilm growth and maturation over four days on large surface area (225 mm^2) conductive ITO:PET substrata was investigated using potassium

ferricyanide as a redox probe [284,285]. Monitoring biofilm over large scale ($\text{mm}^2\text{-cm}^2$) overcomes the local heterogeneity and the statistical issues encountered at smaller areas (μm^2) [286]. Additionally, larger areas allow for the flexible substratum to be bent, curved, or deformed to assume a number of different geometries. We thus investigated the influence of shape and curvature of ITO:PET sheets on the impedance with and without biofilms. The influence of bacterial viability and EPS exopolysaccharides on the biofilm impedance was also investigated by inactivating with sodium azide and carbonyl cyanide m-chlorophenyl hydrazone (CCCP), and using EPS component deleted mutants, Δpel and Δpsl respectively. The electrode reaction processes were understood through cyclic voltammetry. This work presents a proof-of-concept for the rapid detection of a weak electroactive biofilm in the presence of an exogenous redox mediator, and lays out a framework of design considerations for future large surface ITO:PET flexible biofilm sensors.

4.2 Results and discussion

4.2.1 Biofilm growth monitoring via potassium ferricyanide as redox mediator

Here, we used EIS to measure the complex impedance of the biofilm across a broad range of frequencies in presence of 5 mM ferricyanide. Figure 4.1 shows representative Nyquist and Bode plots of EIS measurements of biofilms recorded every 24 hours to match the critical stages of the life cycle of the biofilm. The 0 hour curve in Figure 4.1 corresponds to blank ITO:PET electrode, i.e., an electrode without bacterial inoculum. Both the real and imaginary components of the impedance decreased with growth time of the biofilm. Representative EIS data plotted as a Nyquist plot (Figure 4.1a) showed a depressed semicircle shape, which indicated non-ideal capacitance [219].

Figure 4.1: a: Representative Nyquist plot with respect to bacterial growth time b: Representative Bode phase plot with respect to bacterial growth time

Representative Bode-phase plots (Figure 4.1b) shows the phase angle with respect to frequency. A gradual increase in the phase angle at the lower frequency regime was observed. In contrast, no appreciable changes in the impedance at either medium or high frequencies were observed. We also noted that the phase angle never reached -90° in all measurements, which suggested that large surface ITO:PET-electrolyte interfaces behaved as a CPE [219]. This was in agreement with the depressed semi-circular feature observed in the Nyquist plot.

Here, the frequency regimes indicated the characteristic time of different electrochemical effects. EIS at low frequencies (30 mHz to 3Hz) represented charge transfer and double layer capacitance at the ITO:PET-electrolyte interface. In contrast, the feature at medium (3 Hz to 1 kHz) frequency represented the charge accumulation effects on the ITO:PET-electrode interface as seen in Figure 4.2. In presence of ions in the electrolyte, two time-constants were observed whereas, only one time constant was apparent in absence of any free ions (water) indicating that the ions accumulated near the electrode leading to the time constant at medium frequency. The signal at high (1 kHz to 100 kHz) frequencies was attributed to the resistive behaviour of the electrolyte solution to the applied AC signal [218]. Accordingly, the medium

and high frequencies in figure 4.1b were influenced by the ionic concentration in the system and therefore did not show any significant changes [219].

Figure 4.2: Bode phase plot of blank ITO:PET in various electrolytes

To study the impedance components at each frequency, the DFRT of the ITO:PET-biofilm system was calculated. Generally, DFRT curves comprise of distinct peaks that correspond to the different electrochemical phenomena in the system [287]. The location and shape of the peaks indicate the characteristic time of said phenomena.

Figure 4.3: a: Representative DFRT plots for increasing growth time b: Equivalent circuit

Figure 4.3a shows representative DFRT plots for the various growth times. We observed two distinct peaks at approximately 1 s and 0.01 s. For the blank ITO:PET, the contribution of low

frequency behaviour to the total impedance was approximately 20 times larger than the medium frequency behaviour. The area under the peaks at low relaxation time (0.01 s) did not change with the biofilm growth, while the area under the peaks at 1 s decreased with biofilm growth and maturation. The large difference between these two contributions indicated that the low frequency regime was dominant, and therefore we focused our future analysis on the behaviour at these frequencies.

The changes in impedance at the low frequency region of the EIS plots observed in the DFRT analysis were due to changes in: (i) charge transfer resistance (ii) diffusion related resistance, and (iii) double-layer capacitance [196]. The charge transfer resistance corresponded to the charge exchange at the semiconductor-electrolyte interface to equilibrate the redox potential of the ferricyanide and the Fermi energy of ITO, whereas diffusion related resistance was a result of mass transport of ferricyanide to the electrode in order to reduce to ferrocyanide [219,288]. ITO, an n-type degenerate semiconductor, formed a space charge layer in response to EIS [289]. The accumulation of the majority charge carriers in the space charge region gave rise to the capacitance at the low frequency [290]. As CPE was used for the modelling, capacitance was calculated using Hsu-Mansfeld equation. This equation was applicable for our case because (i) CPE and resistance were parallel in equivalent circuit, (ii) normal distribution of time constant were observed in DFRT, and (iii) CPE coefficient, α ranged between 0.65 and 1 [227,291]. It is to be noted that the margin of error for capacitance calculation were higher for values with $\alpha < 0.7$.

Since the DFRT analysis showed two distinct time constants, a Randles equivalent circuit with two resistor-CPE circuits (Figure 4.3b) was chosen to represent the electrochemical system, with one additional resistor in series to account for the resistance in the solution [215]. Here, charge transfer and diffusion related effects, (from here on known as interfacial resistance) at electrode-biofilm interface were combined to simplify the circuit and apply Hsu-Mansfeld

equation [219,263]. Moreover, as no change was observed at the high and medium frequency in figure 4.1b, the following results describe the features of the time constant at low frequency.

Figure 4.4 shows the interfacial resistance and effective capacitance from the fitted EIS results with respect to the biofilm growth time. The interfacial resistance decreased substantially compared to blank ITO:PET in the first 24 hours. This marked drop in interfacial resistance indicated the potential to detect the initial attachment and formation of biofilms. Although no significant difference was observed between 24 and 96 hours (Figure 4.4a), a decreasing trend was evident. Additionally, there was an evident transition from a more variable decrease in interfacial resistance at 24 hours to a robust decrease at 96 hours (as seen from the scatter plot), which suggested that the electrochemical signature of the biofilm was more repeatable for more developed biofilms on the ITO:PET substratum. From these observations, we hypothesized that the change in interfacial resistance was correlated to the interactions of ferricyanide with bacteria attached to the electrode.

Figure 4.4: a: Interfacial resistance with respect to bacterial growth time and b: effective capacitance with respect to bacterial growth time. NS = Non-significant; $p < 0.05$

In stark contrast to the interfacial resistance, effective capacitance, extracted from CPE changed minimally with time (Figure 4.4b). No substantial change in capacitance was observed

as the effect of the ITO was dominant and masked the contribution from the biofilm. As a result, ITO:PET was insensitive to the thickness of the biofilm after initial attachment.

In this work, we conducted a frequency sweep across a broad range in contrast to continuous monitoring of *P. aeruginosa* biofilms at a single frequency since the dominant frequency range of EIS behaviour on ITO:PET films has not been studied before [246,247,253]. Our results identified 30 mHz to 3 Hz as the frequency range of interest and the interfacial resistance as the primary marker for the detection of bacterial growth.

4.2.2 Need for an exogenous redox mediator

P. aeruginosa primarily undergoes EET via mediated electron transfer through secretion of redox-active mediators known as phenazines (e.g., pyocyanin) that are produced at various stages of biofilm development [207].

Figure 4.5: a: Representative Nyquist and b: Representative Bode plot with respect to growth time

without external redox probe. c: Interfacial resistance and d: effective capacitance with respect to growth time. NS = Non-significant; $p < 0.05$

Figure 4.5 shows the electrochemical response of phenazines with respect to biofilm growth time. The representative Nyquist plot (Figure 4.5a) and Bode plot (Figure 4.5b) did not show any change in impedance and phase angle respectively. The fitted interfacial resistance (Figure 4.5c) and effective capacitance (Figure 4.5d) corroborated the former claim. Here, the phenazines in our system were either washed away along with the planktonic cells in the cleaning step or not sufficient to drive the EET. In principle, the state of biofilms formed by *P. aeruginosa* may be quantifiable by measuring the concentration of these phenazines. However, phenazines are redox- and photo-sensitive and are a by-product of both planktonic and biofilm populations, which makes it difficult to accurately detect it in biofilms [209].

Figure 4.6: Representative Nyquist plot at open circuit potential (OCP) for 72 hours old *P. aeruginosa* biofilms in the presence of different redox mediators: no mediators (black curve), 50 μM HNQ (red curve), 10 mM $[\text{Fe}(\text{CN})_6]^{3-}$ (blue curve).

To improve EIS sensitivity, an exogenous redox mediator was tested to enhance the electron transfer rate towards the electrode through ionic migration and conduction [210]. For example, ferrocene/ferrocenium couple, ferricyanide/ferrocyanide couple, and quinone derivatives have

a redox potential suitable for bio-electrochemical processes (i.e., below 0.7 V vs. standard hydrogen electrode (SHE)) and have been previously reported to detect EET at the biofilm/electrode interface [211–213,292]. Therefore, ferricyanide ions and 2-hydroxy-1,4-naphthoquinone (HNQ) were tested as a redox mediator to enhance the electron transfer rate towards the electrode through ionic migration and conduction [210,212,293]. However, addition of ferricyanide showed better EIS characteristics and was therefore used for subsequent experiments (Figure 4.6).

Figure 4.7: a: Representative Nyquist and b: Representative Bode plot with respect to ferricyanide ions concentration. c: Interfacial resistance and d: effective capacitance with respect to ferricyanide ions concentration. NS = Non-significant; $p < 0.05$

The role of ferricyanide on EET was determined (Figure 4.7) on mature biofilms (72 hours) at three different concentrations (1 mM, 5 mM, and 10 mM). Figure 4.7a shows the representative impedance data plotted as a Nyquist plot. As the concentration of redox mediator increased,

the impedance of the biofilm decreased. Figure 4.7b showed a decrease in phase angle at the lower frequency regime as well as at the lower end of the medium frequency regime. The changes at the medium frequency were due to varied charge accumulation [294] whereas, the lower frequency regime response was due to increased interaction of ferricyanide ions with the electrode. Figure 4.7c,d summarizes the interfacial resistance and effective capacitance at increasing ferricyanide concentration. The interfacial resistance decreased considerably at higher ferricyanide concentrations. As ferricyanide ions diffused through the biofilm, bacterial cells facilitated EET by interacting and reacting with the ferricyanide ions. These interactions occurring in the biofilm were primarily due to diffusion driven EET and the diffusion of redox active ferricyanide ions to the bacteria-electrode interface [295].

For effective capacitance, a smooth increase was observed till 5 mM whereas, a wide scatter plot was observed at 10 mM indicating that the response was highly sensitive to the concentration of ferricyanide. This was expected because the biofilm capacitance is primarily governed by the ionic concentration of an electrolyte at the electrode [196]. By increasing the concentration of the ferricyanide the diffusional layer thickness decreased, thereby resulting in increased capacitance.

5 mM was considered the optimal concentration of ferricyanide after testing the three concentrations on biofilms grown for 72 hours due to its stability in both interfacial resistance and effective capacitance response. This concentration was deemed safe as we were unable to observe any toxicity from 5 mM ferricyanide on the biofilms (Figure 4.8). Here, we did not observe drastic cell death after exposure to 5 mM ferricyanide solution. The dead cells observed after ferricyanide exposure were considered irrelevant since, dead cells were also present in the sample not exposed to ferricyanide. It is to be noted that the before and after samples shown are not biological replicates.

Figure 4.8: Live/Dead CLSM images of 72 hours grown biofilm without (top) and with (bottom) ferricyanide ions. Scale bar represents 10 μm .

4.2.3 Reaction kinetics at the biofilm-ferricyanide interface

We explored the cyclic voltammetric response of the biofilms across 4 days of growth at 5 different scan rates to understand the nature of reactions occurring at the biofilm-electrode interface that affect the impedance characteristics. Cyclic voltammetry is comparatively well-defined to study the kinetics of electrode reaction processes [283]. Figure 4.9a shows the representative cyclic voltammogram at 50 mV/s for varying growth times in the presence of 5 mM ferricyanide ions. Figure 4.9b,c summarizes the peak current and potential at all scan rates and growth times. Under reversible conditions, peak current varied linearly with the square root of scan rate however, the peak potential was independent of scan rate [196]. In our observations, the shape of the plots (Figure 4.9a) was stretched out as compared to a typical symmetric cyclic voltammogram [296]. Additionally, a large peak separation was present. Although, a linear relationship between peak current and scan rate (correlated to reversible

conditions) (Figure 4.9b) was observed, the linear dependence of peak potential on scan rate (Figure 4.9c) suggested sluggish kinetics at the biofilm-electrode interface [197,297]. All these factors indicated an occurrence of a quasi-reversible redox reaction at the electrode where the reaction was controlled by both EET and mass transport of charge [296]. Moreover, the peak anodic and cathodic current at various scan rates summarized in Figure 4.9b showed minimal change in current with respect to increasing growth time. Although the change in current was not apparent, a shift in bias potential was observed for biofilms grown for 72 h and 96 h in both representative cyclic voltammogram (Figure 4.9a) and $\log(\text{scan rate})$ vs the peak bias potential (Figure 4.9b) indicating a clear change in the kinetics at higher growth time. From these plots, it was also evident that redox peaks appeared even in absence of bacteria on the ITO:PET supporting the previous claim that ITO:PET interacted with the ferricyanide ions (see Figure 4.4b).

Figure 4.9: a: Representative cyclic voltammogram at 50 mV/s for various growth times b: Scan rate and current relationship following Randles-Sevcik equation c: Scan rate and peak potential relationship according to Laviron equation

Despite the quasi-reversibility, the Randles-Sevcik behaviour to understand the diffusion characteristics applies in our case due to the linear relationship between peak current and square root of scan rate [298,299]. This was further used to calculate the electrode surface area after bacterial attachment. The diffusion coefficient of the system was calculated for the uncolonised electrode as $7.09 \times 10^{-8} \text{ cm}^2/\text{s}$ at 50 mV/s scan rate. Here, the diffusion coefficient in our case was lesser than the values cited by the literature because of the probable decrease in diffusion layer [196]. Assuming that the diffusion coefficient remained constant despite bacterial attachment, the calculation of electrode surface area was done as per previous report [212].

Figure 4.10: a: Surface coverage as calculated from Randles-Sevcik equation (line at $y = 2.25$ represents blank ITO:PET surface area) b: Reduction and c: Oxidation rate constant as calculated from Laviron equation. NS = Non-significant; $p < 0.05$

Figure 4.10a shows an increase in the electrode surface area after bacterial attachment across 4 days. The line at $y = 2.25$ indicated the surface area of a blank electrode. Similar to EIS, no significant change was observed at different phases of biofilm growth [294]. Furthermore, the increase in surface coverage after bacterial attachment suggested that ITO:PET and biofilm together participate in reactions at the electrode.

The rate kinetics of these redox reactions at the electrode were derived from the trumpet plot seen in Figure 4.9c [300]. Since, the peak separation ($E_{pa} - E_{pc}$) was greater than 200 mV, a linear regression of the trumpet plot was used for the calculations, as dictated by Laviron equation [264]. Figure 4.10b,c shows the change in redox kinetics as a function of growth time. Here, the reduction reaction (Figure 4.10b) was more significant than the oxidation reaction (Figure 4.10c) leading to the sluggish kinetics. Moreover, it suggested that the reaction favored the reduction to ferrocyanide [301]. A statistically insignificant increase in the rate of the reduction reaction was observed after 72 hours of the growth whereas, the rate dropped after 96 hours. The huge standard deviation was due to variability in the biofilm formation.

Although ferricyanide-ITO redox reactions were dominant in the system, the surface coverage and kinetics data suggested that biofilm facilitated the redox reactions at the electrode. Such reactions have been described for *S. oneidensis* in the past. We extended the reactions to *P. aeruginosa*-ferricyanide system as follows [302]:

1. $K_3Fe[C�]_6 \longrightarrow 3K^+ + Fe[C�]_6^{3-}$ (Ionization)
2. $Fe[C�]_6^{3-} (\text{bulk}) \longrightarrow Fe[C�]_6^{3-} (\text{interface})$ (Transport from bulk to interface)
3. $(\text{cell})_r + Fe[C�]_6^{3-} \longrightarrow (\text{cell})_o + Fe[C�]_6^{4-}$ (Reaction at biofilm interface)

4. $\text{Fe}[\text{CN}]_6^{4-}$ (biofilm) $\longrightarrow$ $\text{Fe}[\text{CN}]_6^{4-}$ (electrode) (Transport to ITO:PET)
5. $\text{Fe}[\text{CN}]_6^{4-} \rightleftharpoons \text{Fe}[\text{CN}]_6^{3-} + \text{e}^-$ (Reaction at ITO:PET)

Yet, both the reaction rate and the current characteristics might also be influenced by the mass transfer of redox probe and capacitive characteristics leading to the large standard deviations [194,216]. Therefore, charge transfer resistance element of the EIS is the most representative electrochemical marker in such systems to study the changes in biofilm for future studies.

4.2.4 Mechanism of biofilm sensing

Although we observed a difference in the resistive parameters at the electrode-biofilm interface after bacterial attachment, currently, there is limited knowledge of the *P. aeruginosa* component that participates in the electrochemical interactions with ferricyanide. Both intracellular, (e.g., *Pseudomonas* cytochrome c- 551 oxidase) [303,304] and extracellular components (EPS) (e.g., polysaccharides, eDNA) [244] may have contributed to the EIS behaviour observed, though the key mechanisms remain unknown despite being crucial for understanding the EIS results. Therefore, we further investigated the role of intracellular and extracellular components.

To investigate the role of intracellular components, the biofilms were treated with energy production inhibiting chemical reagents that render the biofilms metabolically inactive. Two different types of energy inhibitors, NaN_3 and CCCP were studied to account for different energy production mechanisms. NaN_3 is a respiration inhibiting biocide that binds to the cytochrome oxidases in *P. aeruginosa* [246]. This binding ceases the energy production via oxidative phosphorylation. CCCP is a proton motive force inhibitor that blocks energy production in biofilms through disruption of the membrane potential [305].

EIS at OCP was measured for the NaN_3 and CCCP treated samples and compared to the behaviour of blank ITO:PET and their respective controls. The EIS results were fitted using

the same two time constant equivalent circuit previously shown (Figure 4.3b). We focused on interfacial resistance effects as they were recognized as the biomarker for detection in our previous results. Figure 4.11 shows the charge transfer behaviour for NaN_3 (Figure 4.11a) and CCCP (Figure 4.11b) treated samples. We observed a change in the interfacial resistance between non-colonized and colonized ITO:PET sheets in both instances. The NaN_3 treatment showed minimal change compared to the control biofilm as seen in figure 4.11a. In contrast, CCCP treatment significantly increased the interfacial resistance (Figure 4.11b). These experiments indicated that there were several intracellular routes for interfacial electron transfer for *P. aeruginosa* biofilms. However, further investigation will be needed to form a complete understanding on the overall EET effects of biocides for *P. aeruginosa* biofilms and such an investigation goes beyond the scope of this thesis.

Figure 4.11: a: Interfacial resistance for blank ITO:PET, control biofilm and NaN_3 treated biofilm b: Interfacial resistance for blank ITO:PET, control biofilm and CCCP treated biofilm c: Current output

at bias potential for blank ITO:PET (N=4, Mean $\pm$ SD), control biofilm (N=6, Mean $\pm$ SD) and NaN₃ treated biofilm (N=4, Mean $\pm$ SD) d: Current output at bias potential for blank ITO:PET (N=4, Mean $\pm$ SD), control biofilm (N=5, Mean $\pm$ SD) and CCCP treated biofilm. NS = Non-significant; $p < 0.05$

Potentiostatic EIS at different potential was used in addition to EIS at OCP to measure faradaic current characteristics for viable biofilms and non-viable biofilms. The application of electrochemical potential drives the EET to occur at a higher rate, and increases the current as a result [223]. This helps differentiate the EET activity of viable and non-viable biofilms. The current output (Figure 4.11c) showed that NaN₃ treatment resulted in lower current output at any potential compared to the control (untreated biofilm), yet, had a slightly higher current output than the blank electrode. For CCCP treatment (Figure 4.11d), the current output for the control biofilm and treated biofilm followed a similar trend as NaN₃ treatment. The difference between the treated sample and blank electrode, however, was insignificant. The variation in the x-axis was due to changes in OCP which was dependent on the electrode surface, biofilm growth, redox reaction kinetics, and the concentration of redox species at the interface [306,307]. Considering the changes after the energy inhibitor treatment in the biofilms, our results confirmed that treatment with inhibitory agents hindered the charge transfer capability of the biofilm.

Extracellular components such as the EPS are another potential contributor to interfacial resistance. To investigate the role of extracellular components, EIS analysis of *P.aeruginosa* mutants with deletion of either *pel* or *psl* exopolysaccharide was conducted.

Figure 4.12a shows no change in the interfacial resistance of WT, Δpel , and Δpsl strains. Bacterial attachment and biofilm development has been shown to happen in the absence of either *pel* or *psl*, which was consistent with the similar interfacial resistance observed in the WT and the two mutants [308] Here, phase of biofilm development may vary for the three

strains considering the similar EIS signature between the initial attachment and the mature biofilm phase as seen in Figure 4.4a. For a better understanding, SPEIS at various potentials was conducted as shown in figure 4.12b. Here, only Δ psl showed a drop in current with respect to WT, while Δ pel current output was similar to WT. This observation was in agreement with previous studies where psl was determined to play a primary role in the biofilm development of non-mucoid PAO1 strains [35,36].

Figure 4.12: a: Interfacial resistance for WT and EPS mutant strains b: Current output at bias potential for WT and EPS mutant strains (N=6, Mean $\pm$ SD). NS = Non-significant; $p < 0.05$

In summary, although significant changes were not observed at OCP, the SPEIS results at higher potential show that EPS components contributed to charge transfer. Despite this, all our studies were done at OCP to avoid the EIS characteristics arising due to faradaic current at the electrode-liquid interface, particularly at low frequency [13].

4.2.5 Role of active surface area on electrochemical response

In addition to the growth of biofilm, the material and the surface area available for biofilm growth may affect the sensitivity and reliability of EIS monitoring. Considering that our ITO:PET substratum was flexible, we next conducted EIS analysis of ITO:PET for different shapes and geometries with and without biofilms present on the surface.

A summarized comparison between blank ITO:PET and biofilm grown for 72 hours is shown in figure 4.13. EIS always measured a significant decrease in the interfacial resistance once a mature biofilm formed after 72 hours, irrespective of shape (Figure 4.13a). Interestingly, the rectangular shape appeared to be the most robust shape for the detection of the biofilm. Figure 4.13b shows the capacitance across the various geometries studied with and without biofilms present. From Figure 4.13b, we observed no significant difference in capacitance across all experimental conditions except rectangle.

Figure 4.13: a: Interfacial resistance with respect to electrode shape and b: effective capacitance with respect to electrode shape. NS = Non-significant; $p < 0.05$

Figure 4.14 and Figure 4.15 shows the interfacial resistance and capacitance obtained from 4 day growth on different shapes of the ITO:PET electrode respectively. It is to be noted that Figure 4.14a and figure 4.15a are same as figure 4.4 and have been added here as reference.

The interfacial resistance of four days of biofilm growth indicated similar trends for square and bent-square (Figure 4.14a,b). The rectangular geometry was able to differentiate between early attachment and mature biofilm (Figure 4.14c). In contrast, the cylindrical shape only measured a significant change in interfacial resistance after 72 hours (Figure 4.14d).

Figure 4.14: Interfacial resistance of a: Square b: Bent square c: Rectangle d: Cylinder. NS = Non-significant; $p < 0.05$

Figure 4.15: Effective capacitance of a: Square b: Bent square c: Rectangle d: Cylinder. NS = Non-significant; $p < 0.05$

The difference in sensitivity and robustness between the rectangular and cylindrical shapes may be due to the differences in active surface area, i.e., the area that interacts with ferricyanide, despite having the same geometrical surface area.

The capacitance for each geometry except rectangle tested did not show any significant change across four days of biofilm growth (Figure 4.15a-d). The change in the capacitance for the rectangular electrode was probably because the biofilm growth blocked the interaction of charges and electrode [229].

4.3 Limitations

There are some limitations of our work. Although phenazines are the redox mediators in *P. aeruginosa*, their concentration in our system was not sufficient to drive the EET therefore, external redox probe was used for the study. The investigation of biofilm sensing mechanism was limited to the role of cytochrome oxidases and exopolysaccharides and the other components that may participate in the electrochemical interactions were not studied. The interactions of blank ITO:PET with ferricyanide ions were dominant in both EIS and cyclic voltammetry thus, masking certain aspects of biofilm-ferricyanide interactions. Despite these limitations, our results presented a step towards detecting biofilms in a more realistic scenario, where larger surface area polymeric substrata of various shapes with non-ideal electrochemical properties are common.

4.4 Conclusion

In conclusion, we have shown that EIS at open circuit potential was able to rapidly detect the static growth of *P. aeruginosa* biofilms across large surface areas of ITO-coated flexible polymeric films over extended periods of time up to 4 days. EIS of *P. aeruginosa* in the presence of potassium ferricyanide on flat squares of ITO:PET indicated a decrease in

impedance of the biofilm with increasing growth time, which was mostly due to the decrease of interfacial resistance with biofilm development whereas the interfacial capacitance dominated by space charge effects of ITO showed minimal changes. Reaction kinetics and current characteristics at bias potential under cyclic voltammetric conditions showed a slight increase in rate constant of reduction reaction and surface coverage of bacteria on ITO:PET electrode respectively. The sensitivity of both EIS and cyclic voltammetry was limited to detecting bacterial attachment on the ITO:PET surface and was not able to differentiate between the attachment and maturation phases. Further investigation of the mechanism for changes in interfacial resistance revealed that the effect was likely due to the combination of increased presence of extracellular components (e.g., EPS) and bacterial metabolic activity. Shape or curvature of the sensor showed no influence on the trend of interfacial resistance and effective capacitance of the ITO:PET after biofilm growth, opening up the potential for bespoke sensors to accommodate non-uniform geometries found in biomedical and industrial applications.

5. Microstructural and biological response of *Pseudomonas aeruginosa* biofilm to focused ultrasound

5.1 Introduction

Bacterial biofilms are microstructured bacterial consortia. These bacterial cells display a high degree of physiological and topographical heterogeneity and grow on abiotic surfaces (e.g., biomedical devices) or biological surfaces (e.g., lung tissue) [309]. Bacteria within biofilms are more tolerant to antimicrobials compared to planktonic bacterial cells due to self-produced biofilm matrix consisting of extracellular polymeric substance (EPS), high bacterial concentration, exchange of genetic information in biofilms, differences in growth states of bacteria across the biofilm, and the expression of genes associated with tolerance or resistance to antimicrobial agents [2,5,6]. Given the increased tolerance of biofilms to antimicrobials, there remains a growing need for more effective antibiotics and new approaches to target biofilm infections. Developing novel antibiotics, however, is costly and time consuming. Thus, there have been increased efforts towards alternative approaches to induce cell death and/or promote dispersion of biofilms [89,310].

One particularly promising approach for biofilm disruption is the use of therapeutic ultrasound, a non-invasive and cost-effective technique that provides targeted and localized mechanical effects [122]. Past research on the effect of ultrasound on biofilms has widely focused on low intensity ultrasound combined with antibiotics and/or cavitation agents (e.g., microbubbles). This work showed that ultrasound-enhanced antibiotic treatment improved antibiotic efficacy, increased cell death, and reduced biofilm thickness [153]. Interestingly, the application of ultrasound with microbubbles alone under these exposure conditions had minimal to no effect

on biofilm removal [153,154]. In contrast, high intensity focused ultrasound (HIFU) without the addition of cavitation agents has also induced bacterial detachment from the substratum and left behind a patchy biofilm compared to the untreated control [182,184,188]. Though HIFU alone was capable of disrupting biofilms, the biological effect on bacterial signaling molecules and/or cellular activity triggered by ultrasound exposure has not yet been investigated.

Here, we report on the physical and biological effects of HIFU on biofilms formed by *Pseudomonas aeruginosa*. The biofilms used in the study were grown for 72 hours on square ITO:PET sheets. The microstructural effects of HIFU were investigated through live/dead analysis, exopolysaccharide-lectin binding analysis, crystal violet assay, and electrochemical response. Further, we investigated the impact of HIFU on the secondary messenger signaling system, cyclic diguanylate (c-di-GMP), which is a central regulator of the transition from the planktonic state to the biofilm and vice versa [57]. In our study, we used a fluorescent bio-reporter strain of *P. aeruginosa* to quantify the effects of HIFU on biofilm formation and c-di-GMP signaling [257,311].

5.2 Results and discussion

5.2.1 Changes in volumetric and textural parameters of biofilms after HIFU exposure: Biomass

To determine if HIFU induced a microstructural response in the biofilm we quantified the biofilms formed by PAO1 mutant labelled with Cfp. Figures 5.1 and 5.2 describe the qualitative and quantitative analysis of biovolume of untreated control and biofilms exposed to 0.5 MPa to 5.5 MPa. Due to the heterogeneous nature of the biofilms and associated high variation in biomass values between replicates, a biovolume loss of 20% or more was arbitrarily considered as an estimate for the lower limit threshold for biofilm loss due to HIFU. Accordingly, a

significant loss in biovolume, especially at high pressure amplitudes (4.5 MPa and 5.5 MPa), was observed (Figure 5.1f,g; Figure 5.2a). The results were consistent with a previous study on acoustic pressure variation on *Escherichia coli* biofilms where a reduction in CFU at high pressures (~6.2 MPa) was observed [188].

Figure 5.1: Representative confocal image 3D reconstructions of biofilms formed by PAO1 P_{cdrA}::Gfp exposed to HIFU at a: 0 MPa (Sham) b: 0.5 MPa (black) c: 1.5 MPa (red) d: 2.5 MPa (blue) e: 3.5 MPa (green) f: 4.5 MPa (magenta) g: 5.5 MPa (brown). The colour codes represent the corresponding acoustic pressure amplitude as seen in figure 5.2 (Quantification data).

Figure 5.2: a: Change in biovolume with respect to acoustic pressure amplitude, NS = Non-significant; $p < 0.05$ b: Change in porosity with respect to biovolume c: Change in X/Z aspect ratio with respect to biovolume

Additional volumetric parameters such as porosity and aspect ratio, gave a better understanding of the mechanical changes in biofilms after HIFU exposure [312]. The volumetric properties revealed increase in porosity (Figure 5.2b) and decrease in aspect ratio in X vs Z direction (Figure 5.2c) with respect to loss of biovolume on the substratum. The increase in porosity evidenced by the 3D reconstructions and quantification of biofilms indicated that HIFU penetrated the biofilm and uprooted the cells from the base of the substratum. Furthermore, the change in the aspect ratio of the run lengths confirmed that HIFU distorted the microcolonies in z-direction [258].

Although, both 4.5 MPa and 5.5 MPa indicated changes in microstructure, only 4.5 MPa was considered in the subsequent experiments. This was because the frequency of entire cell removal from the substratum was more after 5.5 MPa exposure. In such an instance, no experimental value was derived from the results.

5.2.2 Influence of HIFU on biofilm matrix

Biofilms are comprised of cells as well as the extracellular matrix that holds them together and thus, biofilm control strategies may affect one or both components of the biofilm. As suggested by previous results, HIFU was able to disrupt the biomass yet its influence on the viability and the biofilm matrix remains unclear. Therefore, the effects of three pressure amplitudes on both the components were studied using confocal imaging, crystal violet assay, and electrochemical monitoring.

Figure 5.3 shows 3D reconstructions of confocal images of biofilms exposed to HIFU and the subsequent effect of HIFU on cell viability marked by live/dead staining. Based on 3D reconstructions (Figure 5.3a-d) and image quantification (Figure 5.3e) of the untreated biofilm and HIFU treated biofilms, there was no significant effect of HIFU at 0.5 and 2.5 MPa on live biofilm biomass. When the pressure was increased to 4.5 MPa, there was a reduction in live

biomass. Interestingly, the dead biomass was also reduced alongside the live biomass especially after exposure at 4.5 MPa (Figure 5.3f). The biofilm detachment by HIFU was in agreement with previous studies [182,184,188]. Additionally, there was no drastic increase in the dead cells after HIFU exposure; cell death was not a primary consequence of HIFU in contrast to the effects observed in presence of external agents such as microbubbles and antibiotics [112,122,153]. Our results therefore suggest that acoustic intensity was linked to the physical disruption of biofilms.

Figure 5.3: Representative 3D reconstructions of confocal images of biofilms formed by PAO1 Gfp stained with SYTO9, PI and exposed to HIFU at a: 0 MPa (Sham) b: 0.5 MPa c: 2.5 MPa d: 4.5 MPa. Note: Green = live cells, Red = Dead cells; e: Change in live cells biovolume f: Change in dead cells biovolume. The shading around linear regression represents 95% confidence interval.

Next, we investigated the effect of HIFU treatment on the distribution and quantity of polysaccharides that comprise part of the *P. aeruginosa* biofilm matrix (Figure 5.4). In addition to the bacterial cells, the biofilm matrix forms a major part of a biofilm [2]. Previously, it has been suggested that low intensity ultrasound significantly affected polysaccharide synthesis expression in *Staphylococcus epidermidis* and *P. aeruginosa* biofilms [163,167]. A positive correlation between EPS (stained by con-A) and biomass loss after low intensity ultrasound exposure in presence of microbubbles has also been determined [172].

In our study, con-A and CWR stains were used to study the role of HIFU on biofilm exopolysaccharides [313]. These are general lectin-based polysaccharide stains and have been previously used to study exopolysaccharides in both biofilms [313] and aerobic granules [314].

In principle, each monosaccharide unit in the biofilm carbohydrates form α and β glycosidic linkages after bonding with another monosaccharide or molecule (for e.g., lectins). Here, α and β linkages are stereoisomers where, the α -glycosidic bond is formed when the binding carbons have the same stereochemistry and β -glycosidic bond occurs when the two carbons have different stereochemistry. The biofilm exopolysaccharides are rich in such α and β linkages that bind to con-A and CWR, respectively [313].

In *P. aeruginosa*, biofilms formed by the non-mucoid PAO1 strain primarily consist of two exopolysaccharides namely, Pel, a glucose rich polysaccharide and Psl, a pentasaccharide repeat structure consisting of mannose, glucose and galactose [32]. Here, con-A has a binding specificity towards α -mannose and α -glucose [48]. Similarly, *P. aeruginosa* consists of cellulose-resembling β -1,4 and β -1,3 glucose units that binds with CWR [315]. To this effect, con-A and CWR stains are not specific to Pel or Psl, rather they give a combined response of both the exopolysaccharides by bonding with α and β -glycosidic link in the structure of the sugar.

Based on 3D reconstructions of the biofilm (Figure 5.4a-d) it was observed that the bacterial cells (blue) were always surrounded by the α -PS (red) at the bottom and β -PS (yellow) on top irrespective of the HIFU pressure applied. Quantitative image analysis (Figure 5.4e) indicated a positive linear relationship between relative α -PS biovolume and the remaining biofilm cells. This observation suggested that α -PS were removed with the bacterial cells after HIFU exposure. In contrast, the relative β -PS volume was less reduced, suggesting that β -PS may remain in the biofilm micro-environment irrespective of cell biomass loss (Figure 5.4f).

Figure 5.4: Representative 3D reconstructions of confocal images of biofilms formed by PAO1 *PcdrA::Gfp* stained with Con-A, CWR and exposed to HIFU at a: 0 MPa (Sham) b: 0.5 MPa c: 2.5 MPa d: 4.5 MPa. Note: Blue = Biofilm cells, Red = Con-A, Yellow = CWR. e: Change in α -polysaccharides biovolume f: Change in β - polysaccharides biovolume with respect to cell biovolume.

The shading around linear regression represents 95% confidence interval.

The observations revealed that HIFU was able to remove the bacterial cells and the α -polysaccharides linked to them, whereas removal of β -polysaccharides located at the top of the biofilm did not depend on the biovolume removal. This indicated two possible effects of HIFU on α -polysaccharides: (i) HIFU interacts with biofilm exopolysaccharides and degrades them or (ii) they are simply removed from the surface along with the bacterial cells due to their strong linking as a result of active or passive dispersal; both resulting in destabilizing the biofilm microstructure [22,172]. Based on the aforementioned indication, con-A bonded with two major glycosyl units in *P. aeruginosa* i.e., mannose (formed ~20% of Psl structure) and glucose (dominant in Pel and formed ~13% of Psl) [52,316]. As a result, the disruption of α -polysaccharides suggested the ability of HIFU to affect both Pel and Psl exopolysaccharides in *P. aeruginosa*.

Although we investigated the effect of HIFU on cells and EPS sugars, these changes alone do not hint at total biofilm removal because the microstructure of a biofilm is dynamic and complex [2]. Therefore, to further understand the relationship between HIFU and total biofilm dispersal, we used a common method for quantifying acoustic-biofilm interactions, crystal violet assay [166–168,317]. Crystal violet stained the entire biofilm i.e., live cells, dead cells, and extracellular matrix [274]. The crystal violet assay (Figure 5.5a) indicated no apparent effect of HIFU at any given pressure on the crystal violet absorbance.

Figure 5.5: a: Crystal violet absorbance at 550 nm for biofilms formed by PAO1 Gfp exposed to HIFU at various acoustic pressures. NS = Non-significant; $p < 0.05$ b: Interfacial Resistance (NS = Non-significant; $p < 0.05$) and c: Faradaic current characteristics at increasing bias potential for biofilms formed by PAO1 Gfp exposed to HIFU at various acoustic pressures

We also utilized EIS to characterize changes in the biofilm biomass and structure as it has been found to be an efficient tool to detect the growth and disruption of biofilms on conductive surfaces [227]. The EIS response of *P. aeruginosa* biofilms grown on ITO:PET substrate suggested that interfacial resistance and current characteristics indirectly measured the viability of the biofilm [294]. The current characteristics were also influenced by Psl in the EPS. Here, an insignificant change in the interfacial resistance with respect to the sham (Figure 5.5b) was observed at all pressures. Moreover, there was no significant difference between the current for sham and HIFU treated biofilms (Figure 5.5c), although there was a slight, albeit not statistically significant, increase in current for 4.5 MPa exposed biofilms. The statistically insignificant changes in β - polysaccharides quantification, crystal violet assay, and electrochemistry suggested that biofilm matrix components remained on the substratum.

Another possibility for the insignificant changes in the crystal violet assay and electrochemical monitoring was that the microstructural effects were a localized phenomenon. The beam focus of a 0.5 MHz HIFU transducer is 3 mm in radial diameter, resulting in an exposure area of $\sim 7 \text{ mm}^2$ [265]. Although this was a relatively small area compared to the entire area covered by biofilm (225 mm^2), it was easily visualized using confocal microscopy. Crystal violet assay and EIS, however, analyzed an area that extended beyond the focus of the HIFU beam. Thus, these sampling methods provided insight on the effects of HIFU on regions within and beyond the focus of the HIFU, and thus, suggested that the results we observed were restricted to only biofilm within the acoustic focus of HIFU. This result further confirmed the spatial control of acoustic bioeffects of HIFU.

5.2.3 Response of c-di-GMP signaling to HIFU exposure

From the microstructural results, HIFU removed large regions of the biofilm however, the biological mechanism behind such observation remains undefined. A previous study has suggested that changes in genetic factors such as quorum sensing, protein metabolism, and motility are possible after exposure to low intensity ultrasound [163]. We focused on the impact of HIFU on a key regulatory system that controls biofilm formation and dispersal and is known to be linked to cellular responses to environmental cues such as changes in oxygen, nutrient concentrations, as well as nitric oxide that lead to dispersal [57]. C-di-GMP is a secondary messenger that plays a key role in regulating the shift between planktonic and biofilm bound cells [60]. Moreover, c-di-GMP is crucial in regulating the stress response in *P. aeruginosa* [318].

Figure 5.6: Representative ratiometric (Gfp/Cfp) images of biofilms formed by PAO1 $P_{cdrA}::Gfp$ exposed to HIFU at a: 0 MPa (Sham) b: 0.5 MPa c: 1.5 MPa d: 2.5 MPa e: 3.5 MPa f: 4.5 MPa g: 5.5

MPa. The colour codes represent the corresponding acoustic pressure amplitude as seen in figure 5.7

(Quantification data).

The intracellular c-di-GMP was quantified using a reporter strain that responds to changes in c-di-GMP concentrations by inducing Gfp production from the promoter of the *cdrA* gene [257]. The fluorescent bio-reporter used in this study was specifically developed for the indirect characterization of the c-di-GMP from planktonic bacteria and biofilms using confocal microscopy [257,311]. Previous optimization of the bio-reporter strain showed that the data gathered from the confocal microscope correlated well with the chemical quantification [257]. This correlation confirmed that the bio-reporter used was an efficient indicator of the c-di-GMP levels within the cells in different parts of the biofilms and at different time. Moreover, the c-di-GMP concentration may vary throughout the biofilm due to physiological heterogeneity. Therefore, this characterization technique provided information about the spatial and temporal distribution of c-di-GMP.

The response of the Gfp channel corresponding to the Cfp channel (seen in Figure 5.1) was analyzed. Figure 5.6a-g shows the representative ratiometric images of Gfp per Cfp signal expressed by untreated biofilms and HIFU-treated biofilms. Inspection of images indicated that the ratiometric signal gradually increased at increasing HIFU acoustic pressures amplitudes. Quantitative image analysis (Figure 5.7) of the confocal images revealed the dependence of change in ratiometric signal on the loss of biofilm biomass.

Figure 5.7a-f shows the scatter plot of Gfp/Cfp ratio with respect to remaining cell biovolume at six different acoustic pressure amplitudes. The increase in the ratio was evident in most samples (~ 4 out of 7 samples) exposed at 4.5 MPa and 5.5 MPa especially when the biovolume loss was above the 20% biovolume loss threshold. Statistical significance was not the best way to characterize the changes in these experiments as both cavitation and biofilm growth are subject to have levels of variability.

Figure 5.7: Gfp/Cfp ratio and biofilm biovolume relationship at various pressure amplitudes relative to the sham - a: 0.5 MPa b: 1.5 MPa c: 2.5 MPa d: 3.5 MPa e: 4.5 MPa f: 5.5 MPa. The shading around linear regression represents 95% confidence interval.

To better understand the influence of acoustic pressure amplitude on the relationship between biovolume and density, the data points were fit linearly at individual pressures. At $p = 0.05$, only the slope of 4.5 MPa response was statistically different from zero. All the p values are shown in the respective plots along with the linear equation (Figure 5.7).

Figure 5.8: Variation in the slope of linear regression with increasing acoustic pressure amplitude.

Error bars represent the 95% confidence interval

Figure 5.8 summarizes the slope obtained from the linear regression for various acoustic pressure amplitudes. Here, the slope of linear regression was always less than zero indicating an increase in the Gfp/Cfp even at the lowest acoustic pressure. Nonetheless, a decreasing trend was observed as the pressure increased. In principle, the amount of c-di-GMP per biovolume is independent to the amount of biomass loss due to HIFU if the removal of biofilm is truly non-specific. At low acoustic pressure amplitudes before 3.5 MPa, this principle held true. However, the negative correlation between biovolume and density was significant at 4.5 MPa and 5.5 MPa. The increase in c-di-GMP concentration per remaining biomass at acoustic pressure amplitudes at and above 4.5 MPa corresponded to more consistent biofilm removal. It was therefore possible that the biofilm had c-di-GMP hot-spots that were detectable when ‘weaker’ portions of the biomass was removed.

5.2.4 Mechanism of c-di-GMP changes

The previous section has clearly detailed that HIFU influences wild type biofilms. Yet, for a better understanding it is important to know mechanisms behind such acoustic-biofilm interactions. One possible mechanism for c-di-GMP change entirely relies on mechanical disruption of either the matrix or cells to cause biofilm disaggregation. Alternatively, the cells actively respond to the acoustic stresses from ultrasound to disperse suggesting ultrasound may induce changes in gene expression that result in active dispersal of the bacterial cells from the biofilm [22]. The mechanism of the changes was tested by treating biofilms formed by $\Delta wspF$ mutation that overproduce c-di-GMP. Such biofilms often contain spontaneous mutants that do not disperse as effectively as the wild-type cells and are characterized by small colony variants with wrinkled morphology [64].

A $\Delta wspF$ mutant that overproduced c-di-GMP as a consequence of the mutation was used for the study [64]. Figure 5.9 shows the comparison of biofilm growth by wild type and $\Delta wspF$ strains. Here, the $\Delta wspF$ biofilms both untreated (Figure 5.9c) and HIFU-treated (Figure 5.9d) were approximately four times thicker than the ones formed by wild type strain (Figure 5.9a,b). The biofilms of high structural integrity formed by $\Delta wspF$ strains were a consequence of high c-di-GMP production and increased levels of Pel and Psl polysaccharides [64]. The biovolume quantification (Figure 5.9e) shows that HIFU exposure decreased the biovolume in both strains yet, contrary to wild type that removed cells all the way down to base, in $\Delta wspF$ just layers of cells were removed from the top.

Figure 5.9: 3D reconstructions of biofilms formed by a: wild type strain – untreated b: wild type strain – HIFU-treated c: $\Delta wspF$ strain – untreated and d: $\Delta wspF$ strain – HIFU-treated. e: Quantitative analysis – biovolume of the untreated and treated biofilms (WT untreated : N=13, Mean $\pm$ SD; WT treated : N=7, Mean $\pm$ SD; $\Delta wspF$ untreated and treated: N=6, Mean $\pm$ SD). NS = Non-significant; $p < 0.05$

For the c-di-GMP response, we tested the effect of HIFU induced biofilm removal at 4.5 MPa and subsequent effect on c-di-GMP concentrations (Figure 5.10). Both qualitative (Figure 5.10a-d) and quantitative (Figure 5.10e) analysis of these $\Delta wspF$ mutant biofilms at 4.5 MPa showed no increase in the ratiometric signal compared to wild type biofilms. The response from biofilms formed by $\Delta wspF$ mutation did not show any change in c-di-GMP despite the

removal of bacterial cells. Here, the lack of dispersal suggested that the genetic signal cascade that regulated the dispersal was blocked.

Figure 5.10: Ratiometric images of biofilms formed by a: wild type strain – untreated b: wild type strain – HIFU-treated c: $\Delta wspF$ strain – untreated and d: $\Delta wspF$ strain – HIFU-treated. e: Quantitative analysis – Gfp/Cfp ratio of the untreated and treated biofilms as a function of loss in biovolume. The shading around linear regression represents 95% confidence interval.

In comparison, a clear change was observed in biofilms formed by wild type strain indicating the influence of a biological mechanism as opposed to a purely mechanical one. Moreover, the increase in c-di-GMP in the wild type biofilms after HIFU treatment suggested a slow transition towards PAO1 $\Delta wspF$ biofilm condition. Hence, an understanding of the response of high c-di-GMP producing biofilms towards HIFU was necessary. The lack of c-di-GMP response in PAO1 $\Delta wspF$ biofilms indicated that biofilms may develop resilience to HIFU as they become stronger.

Next, we investigated the genotypic composition of the biofilms by colony morphology analysis. Specifically, we looked for the presence of wrinkled colony morphology variants indicative of high c-di-GMP production due to mutations in the *wsp* pathway (Figure 5.11a).

An increase in the percentage of wrinkled colonies (Figure 5.11b) was observed for 4.5 MPa treated biofilms as the total CFU decreased whereas, the slope of untreated biofilms was not significantly different from zero. This suggested that at the higher acoustic pressure, where loss of the biofilm was observed, the remaining biofilm appeared to have a higher relative abundance of genetic variants that overproduce c-di-GMP. Therefore, it is possible that HIFU induced dispersal of wild type cells, leaving behind the wrinkled variants that overproduced c-di-GMP. This may explain the observations above (Figure 5.7) where we observed an increase in relative c-di-GMP production after exposure to increased acoustic pressures.

Figure 5.11: a: Representative images of various colony variants found in the biofilms formed by PAO1 $P_{cdrA}::Gfp$ after exposure to HIFU at 4.5 MPa. b: Relationship between total CFU and % of wrinkled colonies after sham and 4.5 MPa HIFU treatment. The shading around linear regression represents 95% confidence interval.

The results clearly indicate increase in c-di-GMP concentration in a biofilm and several consequences of such change have been previously reported. In *P. aeruginosa*, changes in c-di-GMP resulted in functional changes in LapG, which then cleaved *cdrA* from the surface of the cell. Since Pel was also attached to *cdrA*, this polysaccharide was then also released from

the cell surface to enable dispersal [67]. Moreover, increased c-di-GMP levels were also shown to inversely affect the quorum sensing regulated *rhl* and *pqs* systems [319]. Thus, it is plausible that biofilms have a biological response to the mechanical stress from HIFU.

5.2.5 Role of HIFU parameters and properties in microstructural/biological changes in biofilms

To observe any effects, the choice of acoustic parameters is crucial [185]. At low acoustic intensity, exposing *P. aeruginosa* biofilms to varying ultrasonic frequency did not show any significant change in biofilm viability [159]. In contrast, previous studies at high intensity have shown that varying parameters such as duty cycle, burst period and exposure time of HIFU cause loss of biomass in *P. aeruginosa* and *Enterococcus faecalis* biofilms [182,184]. All of these studies revealed a vital role of acoustic intensity in the disruption of biofilms. Furthermore, the mechanical effects of HIFU such as cavitation and acoustic streaming that might drive the disruption process rely strongly on the peak negative pressure [123]. However, investigation on the role of varying acoustic pressure amplitude on biofilms is limited [320]. Since our aim was to determine the influence of ultrasound alone on the biofilm we used HIFU to achieve the desired effects. Introducing a coupling cone to the experimental system exhibited more intense power at the focus (Figure 5.12a) compared to free-field (Figure 5.12b).

Figure 5.12: Pressure distribution of 0.5 MHz transducer in a: free-field and b: in presence of a coupling cone sealed with Mylar

The observed effects caused by HIFU at higher pressures may be due to cavitation leading to fluid streaming effects and acoustic radiation forces [125]. The correlation between acoustic properties and maximum microstructural and biological response was apparent (Figure 5.13). An increased inertial cavitation energy was observed at 2.5 MPa and higher when, biofilms were grown on the ITO:PET sheet as opposed to a blank ITO:PET sheet in presence of ABTG medium. Here, the rise in energy at 4.5 MP and 5.5 MPa for blank ITO:PET was probably due to gassy ABTG medium in the chamber. Therefore, at this point, whether the biofilm acted as a nuclei or a cavitation event in the medium cascaded remain unclear.

Figure 5.13: Comparison of cavitation energy on blank ITO:PET and biofilm at various acoustic pressures (N=3, Mean $\pm$ SD)

For the frequency content, the raw data obtained from the oscilloscope was post processed and converted to a power spectral density (PSD) curve. The post processing of acoustic emissions to obtain power spectral density curve has been previously described [321]. Briefly, the post-processing involved extraction of power content from the fast Fourier transform of the received voltage signal. The PSD differentiated between the presence of harmonics and broadband

signal [120]. The presence of harmonics, sub-harmonics, and hyper-harmonics are typical for non-inertial cavitation whereas, broadband noise is usually indicative of inertial cavitation.

Figure 5.14: Inertial cavitation response: broadband signal at a: 0.5 MPa b: 1.5 MPa c: 2.5 MPa d: 3.5 MPa e: 4.5 MPa f: 5.5 MPa.

Figure 5.15: Stable cavitation response: Harmonic signal at a: 0.5 MPa b: 1.5 MPa c: 2.5 MPa d: 3.5 MPa e: 4.5 MPa f: 5.5 MPa.

Inertial cavitation response was observed at 3.5 MPa and higher, in the representative PSD (Figure 5.14). It is to be noted that this analysis just described inertial cavitation and there was always a possibility of non-inertial cavitation which was often revealed in the frequency content of the unfiltered signal. Investigations of unfiltered data revealed harmonic signal at higher pressures indicating stable cavitation due presence of bubbles near or within biofilm (Figure 5.15).

Emergence of a non-linear acoustic wave may be another phenomenon affecting the biofilms [130]. In contrast to the wave propagation in free-field (Figure 5.16a), the distortion of rarefactional acoustic waves above 4.5 MPa indicating non-linear wave propagation was evident in a coupling cone setup (Figure 5.16b).

Figure 5.16: Acoustic wave propagation at various acoustic pressures in a: free-field and b: in presence of a coupling cone sealed with Mylar

In summary, the results showed an influence of both cavitation and propagation wave non-linearity on the biofilm response. Previously, the application of external shear in the form of fluid flow has shown increase in the c-di-GMP levels in planktonic *P. aeruginosa* and was associated with increased biofilm development [61]. Thus, it is possible that HIFU was sensed through similar mechanosensing mechanisms. However, studies on bacterial mechanosensing

are limited to the planktonic cells and its effect on biofilm is unknown [322]. It has been suggested that either changes in rotation of the flagella or the membrane protein PilYI may act as mechanosensors in *P. aeruginosa* and further work is needed to determine if these bacterial components are also involved in HIFU sensing [323,324].

5.3 Limitations

As the characterization techniques used were destructive, a before and after effect of ultrasound on the same sample was not possible. Instead, an untreated biofilm grown from the same overnight culture was considered as a control. This control may not exactly resemble the treated sample resulting in variability in our observations. Additionally, there were limitations for the transcriptomic and/or nucleotide analysis of the HIFU-treated biofilms (which may give more insight into the biological response) due to the amount of biomass available in the test system. HIFU is used in biological applications to focus sound waves to create a small volume of intense acoustic energy, thereby avoiding off-target side effects. Here, the focus is not a point in space but a small elliptical volume resembling a grain of rice with maximum intensity at the center. To reiterate, for a 0.5 MHz transducer, the focus has a 3 mm radial diameter [265]. Therefore, we believe that the microstructural and biological changes were localized to this area as was evident from the electrochemical and crystal violet data. Therefore, we are constrained to the volume of biofilm exposed to HIFU to study its effects. This localization amounted to a miniscule volume of bacteria extracted and post-processed for biological characterization thereby, limiting a direct quantification of c-di-GMP levels in the system. As a result, an indirect although efficient fluorescent bio-reporter was used. Future studies (beyond the scope of this report) will explore: (i) other acoustic parameters (duty cycle, HIFU frequency) (ii) different HIFU transducers (for e.g., multi-element transducers with focus of 6 mm radial diameter) (iii) introduction of cavitation agents (for e.g., polymeric nano-cups or

multi-cavity shells) [146,175], and (iv) switching to a relatively dynamic flow-cell system for biofilm growth [325] to address the limitations.

5.4 Conclusion

In summary, acoustic pressures equal to or greater than 4.5 MPa were optimal to observe HIFU-biofilm interactions. The cell viability studies showed that HIFU at 4.5 MPa removed bacteria from the surface although the complete removal was never achieved. Staining the exopolysaccharides revealed that HIFU penetrated the β - polysaccharides and was able to remove and/or degrade α - polysaccharides. Although, biofilm cells were removed from the surface, components of biofilm matrix still remained as observed from crystal violet and EIS studies. The most prominent observation was increase in the c-di-GMP signal after HIFU exposure suggesting that the remaining biofilms have a biological response to HIFU. Therefore, while investigating mechanical/physical approaches on biofilm, looking at the removal of biomass and change in microstructure is not sufficient. It is vital to track the transcriptomic response of the surviving biofilm.

6. Influence of successive focused ultrasound exposures on *Pseudomonas aeruginosa* biofilms

6.1 Introduction

The capability of the biofilms to fight against any external agents that challenge their natural microenvironment is well known. An example of this is the resistance of bacterial biofilms to antibiotic/antimicrobial agents [5,6,80] due to slow penetration of antibiotics [82,83], altered microenvironment, slow growth rate [80], and increase of persister cells [86].

Ultrasound as a bactericidal approach has been validated by various reports [112,122,153,154]. Yet, the development of resistance by surviving biofilm towards ultrasound has been rarely addressed. Multiple ultrasound treatments have been mainly used in *in vivo* systems where delivering the antibiotics/antimicrobials was the primary objective [166,169,170]. Most recently, successive low frequency ultrasound exposure (5 min, every 8 hours) on *in vitro* catheter disk populated with *S. aureus* or *Klebsiella pneumoniae* strains showed a decrease in the CFU relative to single exposure but, portions of viable biofilm still remained [326,327]. Although they used a synergistic approach of antibiotics and ultrasound to tackle the surviving cells, the emerging antibiotic resistance may interfere after multiple dosages in the future. Moreover, the increase in c-di-GMP concentration after HIFU exposure shown in the previous chapter hints that multiple ultrasound exposure may transform the biofilms into a hyper biofilm forming state presented by small wrinkled colony variants [64]. Therefore, a better understanding of the activity of surviving biofilm is required to improve the anti-biofilm strategies.

In this chapter, successive HIFU exposure at 4.5 MPa for 3 min was tested using two protocols. The first protocol examined the planktonic cultures grown from the bacterial cells scraped from

HIFU-treated biofilms and the subsequent biofilms grown from these cultures. The second protocol tested the effect of multiple HIFU exposures on a single biofilm. Finally, a real-time monitoring setup for future experiments was proposed.

6.2 Results and discussion

6.2.1 Ultrasound resistance study 1: Effect on culture and subsequent biofilm growth

The aim of this study was two-fold. First, the growth characteristics of the overnight culture extracted from HIFU treated samples were investigated. Further, the effect on biofilm growth after multiple HIFU treatments was studied.

Figure 6.1: Optical density (Absorbance at 600 nm) of the planktonic cultures formed by the cells extracted from untreated and HIFU-treated biofilms. Here, S = Sham or untreated biofilm U = Ultrasound or HIFU-treated biofilm.

Figure 6.1 shows the optical density (OD) of the planktonic culture during stationary phase of growth curve. Here, the OD of the initial overnight culture was used as a global control. A threshold line was drawn at the minima of the global control and anything below was considered relatively weaker growth. Also, each HIFU treatment had an internal control i.e.,

an untreated biofilm grown from the same culture. After the first round of treatment, the bacteria collected from HIFU-treated biofilm (U) easily recovered and behaved similar to global (OC) and internal control (S). This strongly suggested that the surviving biofilm was viable and prone to recolonization. After biofilm re-growth and second round of treatment, a slight increase in the OD in both 2xUntreated (SS) and 1xHIFU-treated culture (SU) was observed. Interestingly, 2xHIFU-treated (UU) cultures showed a marked decrease in the OD suggesting weak growth of bacteria. A similar behaviour was seen in the internal control (US). These results indicated that weaker biofilms were formed after first treatment despite the planktonic growth recovery. However, after third round of re-growth and treatment, high OD was observed in all cases including 3xHIFU-treated sample (UUU). Overall, these results suggested that the surviving biofilm cells had the ability to re-grow in planktonic conditions and recolonize other surfaces.

The aforementioned results discuss the behaviour of cells under planktonic conditions. Yet, an understanding of the nature of the biofilm grown and the role of multiple HIFU treatment on the surviving biofilm is necessary. For this part of the study, the wrinkled small colony variants corresponding to high c-di-GMP production were counted. Figure 6.2 shows the wrinkled colonies (%) in the total CFU at all iterations. Figure 6.2a confirmed that the initial culture did not contain any wrinkled colonies thereby, it wasn't a source of colonies' emergence in later rounds. After single HIFU exposure an increase in the c-di-GMP activity was observed as the number of wrinkled colonies increased with a decrease in CFU/mL (Figure 6.2b). It is to be noted that this figure is same as figure 5.11b and has been duplicated here for a better understanding of the next figures.

For the second and third round of the treatments, biofilms were grown from both untreated and HIFU-treated cultures of first and second treatment samples respectively. Further, these biofilms were treated with HIFU and an internal control of untreated sample was maintained.

Figure 6.2: The correlation between total CFU (circular plus wrinkled colonies) and percentage of wrinkled colonies at various exposure iterations: a: Round 0; CFU of initial overnight culture b: Round 1; First round of ultrasound treatment c,d: Round 2; Second round of ultrasound treatment and

e,f,g,h: Round 3; Third round of ultrasound treatment. The shading around linear regression represents 95% confidence interval.

Interestingly, whenever the biofilms were left untreated atleast twice (Figure 6.2c, 6.2e, SUS in Figure 6.2f and USS in Figure 6.2g), steep slopes of linear regression indicating random distribution of wrinkled particles (irrespective of the total CFU/mL) were observed. This suggested that the procedure of re-growing the biofilms probably introduced certain variations in biofilms in no particular trend. In figure 6.2d, both untreated (US) and HIFU-treated biofilms (UU) presented CFU/mL lower than other biofilms, indicating the formation of weak biofilms. However, it was in the samples with atleast 2x HIFU-treatment during the third round of treatment, that a peculiar trend was observed (SUU in Figure 6.2f, USU in figure 6.2g and Figure 6.2h). Although c-di-GMP overproducing biofilms (as evident from high levels of wrinkled colonies) were formed, HIFU showed a positive correlation between wrinkled colonies and total CFU/mL. This meant that during the disruption process, HIFU was able to mechanically shear off the stronger counterparts of the biofilms. This was most evident in 3x HIFU-treatment (UUU in Figure 6.2h).

Figure 6.3: Summary of the slopes of linear regression (m) and statistical significance (p) from $m = 0$. The findings representing the slope of linear regression and p-value (indicating the significant difference of the slope from zero) have been summarized in figure 6.3.

The scraping of the cells and re-growth of the biofilm probably added unknown mutations to the biofilm microenvironment thereby, resulting in the observed changes [153]. Yet, from the noticeable difference between untreated controls and HIFU-treated samples we may speculate that multiple HIFU participated to some extent and was able to disrupt the stronger biofilms. The findings were consistent with low frequency ultrasound studies where, multiple exposures reduced the CFU more easily than a single exposure [326,327].

Moreover, it is possible that there was a shift from a biological effect driven 1x HIFU treatment to a mechanical effect driven 3x HIFU treatment. Yet, the reasons for this shift remain unclear.

6.2.2 Effect on a single biofilm after successive HIFU treatment

As another approach towards understanding resistance of ultrasound, the same biofilm was hit with HIFU for 3 days. As a control, a sample grown from the same initial overnight culture was HIFU-treated and analyzed after first exposure using CLSM and colony morphology.

Figure 6.4: 3D reconstructions of biofilms formed wild type strain on a: Day 3 – untreated b: Day 3 – HIFU-treated (1x treatment) c: Day 6 – untreated and d: Day 6 strain – HIFU-treated (3x treatment).

e: Quantitative analysis – biovolume of the untreated and treated biofilms (N=2, Mean $\pm$ SD)

Figure 6.4 shows the qualitative and quantitative decrease in the biovolume after 1x and 3x HIFU-treatment respectively. Here, the 6 day biofilm was approximately 3 times thicker than the 3 day biofilm indicating the formation of a more mature biofilm. Yet, HIFU was able to repeal the cells from the base of the substratum unlike the HIFU effect on strong biofilms formed by $\Delta wspF$ mutations (Figure 5.9c-e).

Figure 6.5: Ratiometric images of biofilms formed wild type strain on a: Day 3 – untreated b: Day 3 – HIFU-treated (1x treatment) c: Day 6 – untreated and d: Day 6 strain – HIFU-treated (3x treatment). e: Quantitative analysis – Gfp/Cfp ratio of the untreated and treated biofilms (N=2, Mean $\pm$ SD). f: Colony morphology analysis of the untreated and treated biofilms (N=2, Mean $\pm$ SD)

Further, the biological effect was studied with CLSM and colony morphology technique. The representative ratiometric images showed a decrease in the Gfp/Cfp ratio after 1x HIFU-treatment (Figure 6.5a,b) and 3x HIFU-treatment (Figure 6.5c,d). Similarly, the quantitative analysis of CLSM images (Figure 6.5e) reflected the decrease in the ratio after 1x HIFU-treatment however, an insignificant change was seen after 3x HIFU-treatment. Furthermore, the colony morphology analysis (Figure 6.5f) showed a statistically insignificant increase in the percentage of wrinkled colony variants for both types of treatment. It is to be noted that

both untreated and HIFU-treated biofilms consisted of negligible number of wrinkled colonies indicating that a thicker but not ‘stronger’ biofilm (at a transcriptomic level) was formed.

The reproducibility issues in the experiments limited the quality of information gathered. Firstly, as the HIFU effect was localized to the acoustic focus, the large surface area of ITO:PET (10 mm diameter) being exposed was probably responsible for non-reproducible results. Moreover, although the initial overnight culture of the biofilm remained the same in control samples, the growth in a separate petri dish may result in a slightly different biofilm leading to the variability in the results. Hence, these studies were repeated only twice. Thus, a control over the area being exposed in addition to monitoring the before and after effects of the same biofilm was necessary.

These limitations maybe addressed by monitoring the ultrasound effect in real time. The following section proposes a design and preliminary results for the real time and continuous detection.

6.2.3 Development of a real time monitoring system to study ultrasound effect on biofilms

Previously, real-time monitoring of biofilm disruption by EIS yielded promising results specifically, a marked change in impedance response after bactericidal agents such as sodium azide [246], bacteriophages [248,249], L-arginine, and antibiotics [247] were introduced to a mature biofilm. The current literature encouraged us to consider EIS as the characterization technique for the real-time monitoring of successive HIFU-treatment effects. For this study, a flow cell setup that catered to both ultrasound equipment and electrochemistry inspired from the previously used static chamber (Figure 2.5) was developed. The development process, experimental considerations, preliminary results and future prospects are discussed in this section.

A commercially available screen printed electrode (SPE) (Metrohm Singapore Pte Ltd, Singapore) i.e., WE, CE, and RE printed on a substratum was considered as the perfect candidate for these experiments (inset of Figure 6.6). For the preliminary tests of the flow cell, carbon SPE was used as a substrata [328]. The prototype of flow cell and idea of the real-time monitoring setup is shown in Figure 6.6. The top part of the flow cell was sealed with a Mylar sheet such that the ABTG medium flow was in between the SPE and Mylar.

Figure 6.6: Proposed design and setup of the real-time and continuous monitoring system. Inset:

Commercially available SPE and the flow chamber

Figure 6.7: Velocity profile of the flow in the sample chamber at various flow rates a: 6 mL/hour b: 12 mL/hour c: 18 mL/hour d: 30 mL/hour

Typically, laminar flow is maintained in the flow cells to allow biofilm growth under controlled and unperturbed conditions [325]. Therefore, to optimize the flow rate, 6 mL/hour to 30 mL/hour were simulated (Figure 6.7). Here, the disturbance of the flow increased with the flow rate. Yet, a uniform flow was observed at the lowest flow rate i.e., 6 mL/hour (Figure 6.7a) and hence, used for the subsequent experiments.

The flow cell system was setup as previously described [325]. Briefly, glass rods connected to silicone tubing were placed in the growth medium. These tubing further connected to the marprene tubing that wrapped the peristaltic pump. The pumping was followed by a bubble trap and the custom-made flow cell. The waste from the flow cell was collected in a separate glass bottle.

Figure 6.8: Electrochemical response to carbon SPE with and without redox probe: a: Charge transfer resistance and b: Effective capacitance

The efficiency of the real time electrochemical monitoring was tested on blank electrodes for 12 hours where, the EIS response was gathered every hour. Since, in absence of bacteria, the interactions of electrode and growth medium are minimal on blank electrode, 5 mM potassium ferricyanide was added to allow electrochemical detection. Figure 6.8a,b show the post-processed fitting values of charge transfer resistance and effective capacitance in presence and

absence of redox probe. The resistance in absence of any redox probe (Figure 6.8a) was extremely high and haphazard due to the absence of any charge transfer elements in the system. However, a decreased and consistent charge transfer resistance was observed when ferricyanide ions were introduced to the system. The decrease in the resistance was due to enhanced interactions between the electrode and ferricyanide ions [219]. Similarly, a huge drop in capacitance was observed when the ionic concentration of the electrolyte was increased although, the values remained consistent over time (Figure 6.8b). This change in capacitance maybe due the transition from a capacitance driven system to a pseudo-capacitive system driven by adsorption of ions, charging-discharging of redox couples, and double layer capacitance [219]. This 12 hour impedance monitoring of the blank electrodes showed that the flow cell system was capable of continuous electrochemical monitoring.

Further, a preliminary test of biofilm growth in presence of 5 mM ferricyanide ions was done. Here, the bacterial inoculum was injected into the flow cell and allowed to deposit for 1 hour before restarting the electrochemical monitoring. Figure 6.9a,b show the equivalent circuits used to analyze the blank electrode and biofilm-electrode combination respectively. The different equivalent circuits indicated introduction of new interfaces after bacterial inoculation [240].

Two intervals of interfacial resistance and effective capacitance were defined where interval A and B correspond to before and after inoculation respectively. The interfacial resistance (Figure 6.9c) remained consistent (albeit with minor disturbances) for the first 24 hours and increased as the biofilm growth progressed. This impedance behaviour was in agreement with the literature [223,224,228,244]. The increased resistance was probably because the biofilm growth restricted the interactions between electrode and ferricyanide [243].

In contrast, the effective capacitance (Figure 6.9d) showed a sudden increase after the bacterial inoculation following which it gradually decreased and stabilized when the biofilm matured. The stable capacitance probably due to full coverage of the electrode was consistent with previous reports on highly conductive electrodes [223,229,232,248].

Figure 6.9: a: Single time constant equivalent circuit for blank electrode b: Two time constant equivalent circuit for biofilms plus electrode c: Interfacial resistance and d: Effective capacitance of biofilm grown for 60 hours. Here, interval A = blank electrode and interval B = after bacterial inoculation.

The effectiveness of the developed flow cell is evident from the preliminary results. Future studies will focus on monitoring the effects of successive ultrasound treatment in real time using this ‘sono-electrochemical’ specific flow cell. For analysis of the acoustic emissions with minimal attenuation, commercialized ITO SPE will be used as substrata for the biofilm growth in the flow cell [329]. Moreover, the optical transparency of the ITO SPE will allow the characterization of c-di-GMP and viscoelasticity of the biofilms in a non-destructive manner as defined by previous reports [37,257].

6.3 Conclusion

On examining the planktonic cultures and subsequent biofilms formed from HIFU-treated biofilms, it was observed that despite the biomass loss, the planktonic cultures were able to recover, multiply, and form a biofilm. A weaker planktonic culture and subsequent biofilm was observed during the second round of treatment however, the biofilm formed from such cultures became even stronger and had high number of wrinkled colonies by third round. Yet, ultrasound was probably not exclusively responsible for the changes and other biological factors may have participated. Therefore, the resistance study on the same biofilm sample was conducted. Here, a negligible change in both Gfp/Cfp ratio and percentage of wrinkled colonies was observed. This protocol presented with a few limitations thereby, increasing the need for a real-time monitoring system. For this purpose, a flow cell housing a screen printed electrode was manufactured and preliminary tests of blank electrode and biofilm monitoring were performed. As the biofilm developed, a gradual increase in the interfacial resistance was observed whereas, a sudden spike followed by stable signal was observed in effective capacitance response. The future prospects of this setup in ultrasound exposure have been discussed.

7. Final conclusions and possible future directions

7.1 Conclusion

This thesis elucidated the microstructural and biological effects of HIFU on biofilms formed by *P. aeruginosa* using CLSM, EIS, crystal violet assay, and CFU analysis. A multi-disciplinary approach covering microbiology, therapeutic ultrasound, microscopy, and electrochemistry efficiently fulfilled certain gaps of knowledge in the current literature. The main conclusions of this work are summarized below.

For the appropriate analysis of the acoustic emissions, electrochemistry, and confocal visualization, ITO:PET was chosen as the biofilm growth substratum. Initial analysis focused on the biofilm growth optimization across 4 days. The biofilm growth visualization using CLSM and crystal violet assay revealed a near-complete coverage of the substratum after 3 days (Chapter 3). Furthermore, EIS, an efficient characterization technique to rapidly detect bacterial cells was optimized to analyze the biofilms grown across 4 days (Chapter 4). Here, EIS was able to respond to the growth of *P. aeruginosa* biofilms across large surface areas of ITO:PET films. A clear change in the interfacial resistance was observed between blank electrode and biofilm coated electrode. In addition, the mechanisms driving the EIS response were investigated. This revealed that EIS was particularly sensitive to viable cells and EPS also played an important role in the electrochemical response.

Further, the effects of ultrasound on *P. aeruginosa* biofilms were investigated in chapter 5. Here, both viable and non-viable cells in addition to few components of exopolysaccharides were removed at acoustic pressures equal to or greater than 4.5 MPa. However, the most prominent observation was that HIFU induced biological effects in the biofilms formed by wild type strain through the increase in the c-di-GMP signal. Yet, stronger biofilms formed due to

$\Delta wspF$ mutations did not show any biological changes after HIFU exposure thereby, hinting that stronger biofilms were resistant to ultrasound.

Multiple ultrasound exposure revealed that by third round of HIFU treatment, stronger biofilms were formed and HIFU still caused microstructural changes. However, ultrasound was probably not the sole contributor to the changes and other biological factors may have interfered. Resistance study on the same biofilm sample showed a negligible change in both Gfp/Cfp ratio and percentage of wrinkled colonies. And the experiments revealed certain limitations in studying multiple HIFU exposure effects. Therefore, a real-time flow cell setup was proposed and preliminary results were discussed. Here, a marked change in interfacial resistance and effective capacitance were observed as the biofilm matured suggesting that changes caused by ultrasound may be viewed in real time.

7.2 Possible future directions

Adapting a continuous monitoring flow cell setup in the future will allow control over the acoustic target and provide new information such as before and after effect on microstructure and c-di-GMP of the same biofilm. Another important and unique property, viscoelasticity will be efficiently characterized in the proposed new system based on a novel characterization developed in our lab (particle tracking micro-rheology). Moreover, the control of the target area will allow gene expression studies from RNA sequencing. This may provide additional information on the influence of HIFU on extracellular matrix, biofilm proteins, c-di-GMP, quorum sensing, and biofilm metabolism.

In the current investigations, the microstructural and biological changes were achieved at tremendously high pressure amplitudes. However, keeping the real life scenario in mind it is preferable to achieve ultrasound induced changes on biofilm at even lower pressure amplitudes with more control over cavitation. This is possible by using cavitation agents which lower the

cavitation threshold. Fabrication and optimization of novel solid cavitation agents is simultaneously being investigated in our lab. Studying the biofilms changes in presence of such cavitation agents coupled with ultrasound is an important branch of the future work.

The work discussed in this thesis and aforementioned prospects open various avenues of combating biofilms in real life applications. In medical applications, where electrically conductive implants are inserted in the body, ultrasound may be applied for treatment and the effects might be simultaneously monitored by EIS. Furthermore, ultrasound may be a promising external stimuli to target antimicrobials to biofilms formed on both organs and artificial implants. The application of HIFU is not limited to biofilms in medical applications. In industrial applications where, biofilms are a nuisance in marine environments, water treatment plants, sewage pipes etc., disruption of biofilms may be achievable by HIFU and cavitation agents (if required).

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Appendix

A.1 Matlab codes

A.1.1 Volumetric parameters determination

Example of Channel 1 calculation:

```
%Define 3D matrix to store processed image stack
%Ch1_Data is the 3D confocal image matrix
Thresh_Filt_Stack = zeros (size(Ch1_Data), 'uint8');

sliceLength = size(Ch1_Data, 3); %z-direction
for slice = 1:sliceLength
    Layer = Ch1_Data(:, :, slice);

    %Threshold graylevel images using inbuilt Otsu's thresholding
    %function from MATLAB Image processing toolbox
    [levelOT] = graythresh(Layer);
    Ch1_Data_thresh = im2bw(Layer, levelOT);
    Ch1_Data_thresh = uint8(Ch1_Data_thresh);

    %Filter binary images using inbuilt 2D median filtering
    %function from MATLAB Image processing toolbox
    Ch1_Data_filt = medfilt2(Ch1_Data_thresh, [5 5]);

    %Assign the processed image to 3D matrix
    Thresh_Filt_Stack(:, :, slice) = Ch1_Data_filt;

end

%Calculate biovolume of processed 3D stack
%Function defined by Lewandowski, Z., and Beyenal, H.
%Fundamentals of Biofilm Research, 2007.
voxVolScaling = info.voxSizeX*info.voxSizeY*10^12 * info.voxSizeZ*10^6;
Biovolume = sum(Thresh_Filt_Stack(:))*voxVolScaling;

%Calculate porosity = total void pixels/total number of pixels
%Function defined by Lewandowski, Z., and Beyenal, H.
%Fundamentals of Biofilm Research, 2007.
Total_pixels = prod(Thresh_Filt_Stack);
Porosity = 1 - (Biovolume/ Total_pixels);

%Calculate run lengths in X, Y and Z directions
%Function defined by Lewandowski, Z., and Beyenal, H.
%Fundamentals of Biofilm Research, 2007.
[AXRL, AYRL, AZRL] = runLengths3D(Thresh_Filt_Stack);
AXRL = AXRL*info.voxSizeX*10^6;
AYRL = AYRL*info.voxSizeY*10^6;
AZRL = AZRL*info.voxSizeZ*10^6;
```

A.1.2 Gfp/Cfp ratio determination

For 3D data quantification:

```

%Define 3D matrices for storage
BIO_Filt_Stack = zeros(size(Ch1_Data)); %For binary biovolume data
Gfp_Stack = zeros(size(Ch2_Data)); %For graylevel Gfp data
Cfp_Stack = zeros(size(Ch1_Data)); %For graylevel Cfp data

sliceLength = size(Ch1_Data, 3);

for slice = 1:sliceLength
    Layer = Ch1_Data(:, :, slice);
    Layer1 = Ch2_Data(:, :, slice);

%Convert to double to allow calculations between two images
    CFPLayer = double(Layer);
    GFPLayer = double(Layer1);

%Create binary mask by thresholding graylevel images
%and filtering binary images
    [levelOT] = graythresh(Layer);
    CFP_Data_thresh = imbinarize(Layer, levelOT);
    CFP_Data_thresh = double(CFP_Data_thresh);
    CFP_Data_filt = medfilt2(CFP_Data_thresh, [5 5]);
    CFP_Filt_Stack(:, :, slice) = CFP_Data_filt;

%Apply mask to Gfp data
    GFP_mask = GFPLayer.*CFP_Data_filt;
    GFP_Stack(:, :, slice) = GFP_mask;

%Apply mask to Cfp data
    BIO_mask = CFPLayer.*CFP_Data_filt;
    CFP_Stack(:, :, slice) = BIO_mask;
end

%Quantify the ratio
    GFP_Ints = sum(GFP_Stack(:));
    CFP_Ints = sum(CFP_Stack(:));
    GFPCFPRatio = GFP_Ints/CFP_Ints;

For qualitative ratiometric images:

%% As described by Nair et al. (2017); doi: 10.1074/jbc.M116.746743
%Create a Max Z-Projection of the Z-stacked image data
    GFP_MaxZ = max(Ch2_Data, [], 3);
    GFP_MaxZ = double(GFP_MaxZ);

    CFP_Max = max(Ch1_Data, [], 3);
    CFP_MaxZ = double(CFP_Max);

%Obtain the ratiometric image
    GFPCFP_RatioImage = GFP_MaxZ./CFP_MaxZ;

%%Auto Threshold the Biomass data with Otsu
    [level1] = graythresh(CFP_Max);
    BIO_OTbw = im2bw(CFP_Max, level1);
    BIO_OTbw = uint8(BIO_OTbw);

%%Filter the image with filter size = 5

```

```

BIO_fl = medfilt2(BIO_OTbw,[5 5]);
BIO_fl = double(BIO_fl);

% Apply binary mask to ratiometric image
GFPBIO_mask = GFPBIO_RatioImage.*BIO_fl;

%Get the final ratiometric image
c = jet(16);
imagesc(GFPBIO_mask);
colormap(c);

```

A.1.3 Power FFT

By: U.S. Jonnalagadda

```

fs=dataToWrite(1,:) * 10^6; %reading Voltage vs. time curve

fs = length(fs);
func= (dataToWrite(2,:) * 10^3) / 5;

%PwrFFT computes the power spectrum
%of a function in the frequency domain

%Use next highest power of 2 greater than or equal to length(x)
%to calculate FFT.
nfft      = 2^(nextpow2(length(func)));

%Take fft, padding with zeros so that length(fftx) is equal to nfft
fftx      = fft(func,nfft);

%Calculate the number of unique points
NumUniquePts = ceil((nfft+1)/2);

%FFT is symmetric, throw away second half
fftx      = fftx(1:NumUniquePts);

%Take the magnitude of fft of x and scale the fft so that it is not
%a function of the length of x
pw_fft    = abs(fftx)/length(func);

%Take the square of the magnitude of fft of x.
pw_fft    = pw_fft.^2;

%Since we dropped half the FFT,
%we multiply mx by 2 to keep the same energy.
%The DC component and Nyquist component, if it exists,are unique
%and should not be multiplied by 2.

if rem(nfft, 2) % odd nfft excludes Nyquist point
    pw_fft(2:end) = pw_fft(2:end)*2;
else
    pw_fft(2:end -1) = pw_fft(2:end -1)*2;
end

% This is an evenly spaced frequency vector with NumUniquePts points.
freq = (0:(NumUniquePts-1))*fs/nfft;
plot(freq/100,pw_fft);

```

```
xlabel('Frequency (MHz)')  
ylabel('Power Spectral Density (mV2/MHz)')
```